

TRANSMITTAL LETTER TO THE UNITED STATES
DESIGNATED/ELECTED OFFICE (DO/EO/US)
CONCERNING A FILING UNDER 35 U.S.C. 371

147-183PCT

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)

09/254870
NEW

INTERNATIONAL APPLICATION NO.

PCT/EP97/05198

INTERNATIONAL FILING DATE

22 September 1997

PRIORITY DATE CLAIMED

23 September 1996

TITLE OF INVENTION

NUCLEIC ACID MOLECULES CODING FOR TUMOR SUPPRESSOR PROTEINS AND METHODS FOR THEIR ISOLATION

APPLICANT(S) FOR DO/EO/US

SPENGLER, Dietmar; JOURNOT, Laurent

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☒ This express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39 (1).
4. ☒ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date
5. ☒ A copy of the International Application as filed (35 U.S.C. 371(c)(2))
 - a. ☒ is transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☒ has been transmitted by the International Bureau.
 - c. ☐ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(3)).
7. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(2)).
 - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
 - b. ☐ have been transmitted by the International Bureau.
 - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
 - d. ☒ have not been made and will not be made.
8. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. ☒ An Information Disclosure Statement under 37 CFR 1.97 and 1.98. /International Search Report
12. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. ☒ A FIRST preliminary amendment.
☐ A SECOND or SUBSEQUENT preliminary amendment.
14. ☐ A substitute specification.
15. ☐ A change of power of attorney and/or address letter.
16. ☒ Other items or information:
 - ✓1.) PCT Request (PCT/RO/101)
 - ✓2.) Deposit of Microorganism and Translation (2 pages)
 - ✓3.) International Preliminary Examination Report (PCT/IPEA/409)
 - ✓4.) Seventeen (17) Sheets of Formal Drawings

U.S. APPLICATION NO (if known, see 37 CFR 1.5) NEW		INTERNATIONAL APPLICATION NO PCT/EP97/05198		ATTORNEY'S DOCKET NUMBER 147-183PCT	
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17. ☒ The following fees are submitted:

BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5):
 Neither international preliminary examination fee (37 CFR 1.482)
 nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO
 and International Search Report not prepared by the EPO or JPO. **\$970.00**

International preliminary examination fee (37 CFR 1.482) not paid to
 USPTO but International Search Report prepared by the EPO or JPO **\$930.00**

International preliminary examination fee (37 CFR 1.482) not paid to USPTO
 but international search fee (37 CFR 1.445(a)(2)) paid to USPTO. **\$760.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO
 but all claims did not satisfy provisions of PCT Article 33(1)-(4) **\$670.00**

International preliminary examination fee (37 CFR 1.482) paid to USPTO
 and all claims satisfied provisions of PCT Article 33(1)-(4). **\$96.00**

ENTER APPROPRIATE BASIC FEE AMOUNT =

Surcharge of **\$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30
 months from the earliest claimed priority date (37 CFR 1.492(e)).

CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total Claims	55 - 20 =	35	X \$18.00	\$	630.00
Independent Claims	2 - 3 =	-----	X \$78.00	\$	-----
MULTIPLE DEPENDENT CLAIM(S) (if applicable) Yes			+ \$260.00	\$	260.00
TOTAL OF ABOVE CALCULATIONS =				\$	1950.00
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28).				\$	
SUBTOTAL =				\$	1950.00
Processing fee of \$130.00 for furnishing the English translation later than <input type="checkbox"/> 20 <input type="checkbox"/> 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				\$	
TOTAL NATIONAL FEE =				\$	1950.00
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40.00 per property +				\$	
TOTAL FEES ENCLOSED =				\$	1950.00
				Amount to be: refunded	\$
				charged	\$

CALCULATIONS

PTO USE ONLY

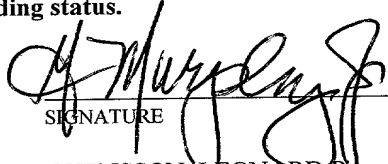
a. ☒ A check in the amount of \$ 1950.00 to cover the above fees is enclosed.

b. ☐ Please charge my Deposit Account. No. _____ in the amount of \$ _____ to cover the above fees.
A duplicate copy of this sheet is enclosed.

c. ☒ The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any
overpayment to Deposit Account No. 02-2448.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

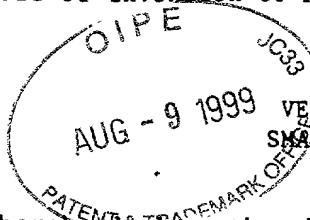
Send all correspondence to:
Birch, Stewart, Kolasch & Birch, LLP
P.O. Box 747
Falls Church, VA 22040-0747
(703)205-8000


 SIGNATURE
SVENSSON, LEONARD R.
 NAME

#30,330
 REGISTRATION NUMBER

/sas March 15, 1999

Applicant or Patentee: Dietmar Spengler and Laurent Journot Attorney's
Serial or Patent No.: 09/254,870 Docket No: 52130-A-PCT-US
Filed or Issued: March 15, 1999
Title of Invention or Patent: Nucleic Acid Molecules Coding For Tumor Suppressor
Proteins And Methods For Their Isolation



VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: Centre National de la Recherche Scientifique

Address of Organization: Rue de la Cardonille, 34094 Montpellier Cedex 05, France

TYPE OF ORGANIZATION:

☒ UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION
☐ TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3)
☐ NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA
NAME OF STATE: _____
CITATION OF STATUTE: _____
☐ WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA
☐ WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA
NAME OF STATE: _____
CITATION OF STATUTE: _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled Nucleic Acid Molecules Coding For Tumor Suppressor Proteins And Methods For Their Isolation

by inventor(s) Dietmar Spengler and Laurent Journot

described in:

☐ the specification filed herewith
☒ application serial no. 09/254,870 filed March 15, 1999
☐ patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below^a and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

^aNOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: Max-Planck-Gesellschaft zur Förderung der Wissenschaften, e.V.
Address: Berlin, Germany

☐ Individual ☐ Small Business Concern ☒ Nonprofit Organization

*See Reverse

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Michel RETOURNA

Title In Organization: Délégué Régional du C.N.R.S.

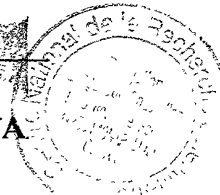
Address: 1919, route de MENDE - 34293 MONTPELLIER CEDEX 5

Signature: _____

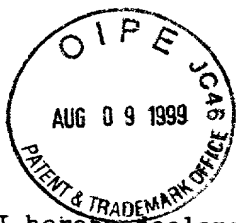
Date Of Signature: 15 juin 1999

Le Délégué Régional,

Michel RETOURNA



Applicant or Patentee: Dietmar Spengler and Laurent Journot Attorney's
Serial or Patent No.: 09/254,870 Docket No: 52130-A-PCT-US
Filed or Issued: March 15, 1999
Title of Invention or Patent: Nucleic Acid Molecules Coding For Tumor Suppressor
Proteins And Methods For Their Isolation



VERIFIED STATEMENT (DECLARATION) CLAIMING
SMALL ENTITY STATUS UNDER 37 C.F.R. §1.9(f)
AND §1.27(d) - NONPROFIT ORGANIZATION

I hereby declare that I am an official empowered to act on behalf of the nonprofit organization identified below:

Name of Organization: Max-Planck-Gesellschaft zur Förderung der Wissenschaften, e.V.

Address of Organization: Berlin, Germany

TYPE OF ORGANIZATION:

X

UNIVERSITY OR OTHER INSTITUTION OF HIGHER EDUCATION

TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3)

NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA

NAME OF STATE: _____

CITATION OF STATUTE: _____

WOULD QUALIFY AS TAX EXEMPT UNDER INTERNAL REVENUE SERVICE CODE 26 U.S.C. §§501(a) and 501(c)(3) IF LOCATED IN THE UNITED STATES OF AMERICA

WOULD QUALIFY AS NONPROFIT SCIENTIFIC OR EDUCATIONAL UNDER STATUTE OF STATE OF THE UNITED STATES OF AMERICA IF LOCATED IN THE UNITED STATES OF AMERICA

NAME OF STATE: _____

CITATION OF STATUTE: _____

I hereby declare that the nonprofit organization identified above qualifies as a nonprofit organization as defined in 37 C.F.R. §1.9(e)* for purposes of paying reduced fees under 35 U.S.C. §41(a) and 41(b), with regard to the invention entitled Nucleic Acid Molecules Coding For Tumor Suppressor Proteins And Methods For Their Isolation

by inventor(s) Dietmar Spengler and Laurent Journot

described in:

X the specification filed herewith

application serial no. 09/254,870 filed March 15, 1999

patent no. _____ issued _____

I hereby declare that rights under contract or law have been conveyed to and remain with the nonprofit organization with regard to the above identified invention.

If the rights held by the nonprofit organization are not exclusive each individual, concern, or organization known to have rights to the invention is listed below^a and no rights to the invention are held by any person, other than the inventor, who could not qualify as a small business concern under 37 C.F.R. §1.9(d)* or a nonprofit organization under 37 C.F.R. 1.9(e)*

^aNOTE: Separate verified statements are required from each person, concern, or organization having rights to the invention averring to their status as small entities. 37 C.F.R. §1.27.

Name: Centre National de la Recherche Scientifique

Address: Rue de la Cardonille, 34094 Montpellier Cedex 05, France

Individual

Small Business Concern

X Nonprofit Organization

*See Reverse

I acknowledge the duty to file, in this application or patent, notification of any change in status resulting in loss of entitlement to small entity status prior to paying, or at the time of paying, the earliest of the issue fee or any maintenance fee due after the date on which status as a small entity is no longer appropriate. 37 C.F.R. §1.28(b)*.

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001, and that such willful false statements may jeopardize the validity of the application, any patent issuing thereon, or any patent to which this verified statement is directed.

Name of Person Signing: Christa Herzog

Title In Organization: Head of patent department

Address: Hofgartenstr. 8, 80539 München

Signature: Christa Herzog

Date Of Signature: München, den 8.7.99

PATENT 147-183PCT

IN THE U.S. PATENT AND TRADEMARK OFFICE

APPLICANT: Dietmar SPENGLER et al
INT'L. APPLN. NO.: PCT/EP97/05198
SERIAL NO.: New GROUP:
FILED: March 15, 1999 EXAMINER:
FOR: NUCLEIC ACID MOLECULES CODING FOR TUMOR SUPPRESSOR PROTEINS
AND METHODS FOR THEIR ISOLATION

PRELIMINARY AMENDMENT

Assistant Commissioner of Patents
and Trademarks
BOX PATENT APPLICATION
Washington, D.C. 20231

March 15, 1999

Sir:

The following Preliminary Amendments and Remarks are respectfully submitted in connection with the above-identified application.

IN THE SPECIFICATION:

Before line 1, insert --This application is the national phase under 35 U.S.C. §371 of prior PCT International Application No. PCT/EP97/05198 which has an International filing date of September 22, 1997 which designated the United States of America.--

IN THE CLAIMS:

CLAIM 5: Line 1, change "any one of claims 2 to 4" to --claim 2--

CLAIM 6: Line 1, change "any one of claims 2 to 5" to --claim 2--

CLAIM 7: Line 1, change "any one of claims 2 to 6" to --claim 2--

CLAIM 8: Line 1, change "any one of claims 2 to 7" to --claim 2--

CLAIM 9: Line 1, change "any one of claims 2 to 8" to --claim 2--

CLAIM 11: Lines 1 and 2, change "any one of claims 2 to 10" to
--claim 2--

CLAIM 12: Line 1, delete "or claim 11"
Line 1 and 2, change "a" to --said-- and delete --as
defined in claims 1 and 11--

CLAIM 13: Line 1, delete "11 or 12"

CLAIM 15: Line 1, delete "or of any one of claims 11 to 14"

CLAIM 18: (AMEND) A nucleic acid molecule of at least 15 nucleotides in length hybridizing specifically with a nucleic acid molecule of claim 1 [or with a nucleic acid molecule of any one of claims 11 to 17] or to a complementary strand thereof.

CLAIM 19: (AMEND) A vector comprising a nucleic acid molecule of claim 1 [or of any one of claims 11 to 17]

CLAIM 21: Line 1, delete "or 20"

CLAIM 24: Line 2, delete "or 23"

CLAIM 25: (AMEND) A polypeptide encoded by a nucleic acid molecule of claim 1 [or of any one of claims 11 to 17] or produced by a method according to claim 24.

CLAIM 40: (AMEND) Use of an effective dose of a nucleic acid molecule of claim 1 [or of any one of claims 11 to 17], or a nucleic acid molecule which is complementary to such a nucleic acid molecule for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of recurrence of a disease in a subject.

CLAIM 42: Line 1, delete "or 20"

CLAIM 45: Line 1, change "any one of claims 40 to 44" to
--claim 40--

R E M A R K S

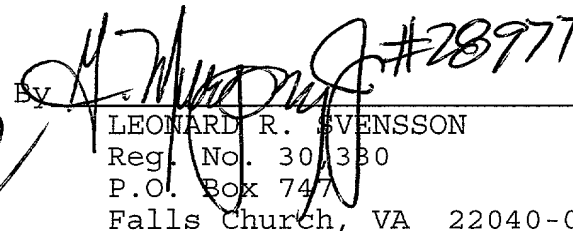
The specification has been amended to provide a cross-reference to the previously filed International Application.

The amendments to the claims is merely to eliminate the improper multiple claims to place the application into better form prior to examination.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §1.16 or under 37 C.F.R. §1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

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17PRTS

09/254870

510 Rec'd PCT/PTO 15 MAR 1999

Max-Planck-Gesellschaft zur
Förderung der Wissenschaften e.V.
Our Ref.: B 2749 PCT

NUCLEIC ACID MOLECULES CODING FOR TUMOR SUPPRESSOR
PROTEINS AND METHODS FOR THEIR ISOLATION

The present invention relates to novel nucleic acid molecules coding for a protein having the biological activity of a tumor suppressor protein. The present invention also provides methods for the isolation of nucleic acid molecules encoding tumor suppressor proteins as well as nucleic acid molecules obtainable by said method. Further, the invention provides vectors comprising said nucleic acid molecules wherein the nucleic acid molecules are operatively linked to regulatory elements allowing expression in prokaryotic or eukaryotic host cells as well as polypeptides encoded by said nucleic acid molecules which have tumor suppressor activity and methods for their production. The present invention further relates to pharmaceutical and diagnostic compositions comprising the aforementioned nucleic acid molecules and/or comprising a nucleic acid molecule which is complementary to such a nucleic acid molecule. Described are also compositions which comprise polypeptides encoded by the described nucleic acid molecules which have tumor suppressor activity and/or an antibody specifically recognizing such polypeptides.

A network of genes, including cell cycle regulatory genes, proto-oncogenes, and tumor suppressor genes have emerged, which play major roles in normal physiological processes as well as in tumor progression (Grana and Reddy, Oncogene 11 (1995), 221-219; Hartwell and Kastan, Science 266 (1994), 1821-1828; Hoffman and Liebermann, Oncogene 9 (1995), 1807-1812; Sherr, Cell 79 (1994), 551-555). Oncogenes have first captured the lion's share of attention in

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the molecular and genetic studies on cell transformation. But it has now been realized that there is an equally important second side of the coin, presented by a distinct class of genes known variously as tumor suppressor genes (TSGs) or anti-oncogenes. Logic dictates that there must exist an equally elaborate array of growth-constraining elements in the cell's signaling circuitry that serve to counteract the growth-promoting proto-oncogenes (Fisher, *Cell* 78 (1994), 539-542; Karp and Broder, *Nature Med.* 1 (1995), 309-320; Liebermann et al., *Oncogene* 11 (1995), 119-210; Thompson, *Science* 267 (1995), 1456-1462). These tumor suppressor genes are of special interest since they may open up new possibilities for the treatment of cancers of various kinds and may help to better understand the molecular mechanisms responsible for the development of cancer.

The isolation of such suppressor genes has become feasible by progress in various fields with major contributions of molecular genetics and cell cycle analysis. Molecular genetics applied linkage studies to the isolation of TSGs, but the most fruitful strategies have evolved from the study of the genetic mechanisms employed by nascent tumor cells to discard their second, surviving copy of a tumor suppressor gene which results in homozygosity at the tumor suppressor locus. This event can often be traced by following the fate of anonymous DNA markers whose polymorphism allows detection of hetero- and homozygous states in these chromosomal regions. By this strategy the identification of the retinoblastoma gene product (Rb), the Wilms tumor suppressor gene (WT) and the von Hippel-Lindau tumor-suppressor gene has been possible. Most recently the cloning of the breast cancer susceptibility genes, BRCA1 and BRCA2 (Miki et al., *Science* 266 (1995), 66-71; Wooster et al., *Nature* 378 (1995), 789-792) has been accomplished by this approach.

Yet, the vast majority of human cancers, including breast cancer, develop spontaneously or under poorly defined criteria of genetic susceptibility preventing linkage studies to perform and indicating that epigenetic mechanisms appear to play the major role in the initiation and formation of tumors, which seem to develop in a multi-step process.

Further support for the concept of TSGs came up with the characterization and isolation of the regulatory components of the mammalian cell cycle. This progress has led to the identification of a new class of candidate tumor suppressor genes, the ubiquitously expressed cyclin-dependent kinase inhibitors (cdk), which negatively regulate cell cycle progression. Among the various forms described so far (p15, p16, p18, p21 and p27) the cdk p16 has been demonstrated to be mutated in-vivo in a spectrum of tumors examined (Marx, Science 264 (1994), 344-345; Kamb et al., Science 264 (1994), 436-440; Nobori et al., Nature 368 (1994), 753-756).

Another important example of a tumor suppressor gene is the p53 TSG, whose biological activity has been elucidated in-vitro through molecular and biochemical studies before it became identified as the genetic cause of the Li-Fraumeni syndrome. It is one of the most frequently mutated tumor suppressor genes in human tumors from various origins (Hollstein et al., Science 253 (1991), 49-53). This TSG encodes a transcription factor with two important functional properties contributing to its growth-suppression function: induction of apoptosis and cell cycle arrest (Vogelstein and Kinzler, Cell 70 (1992), 523-526; Oren, FASEB J. 6 (1992), 3169-3176; Perry, Curr. Opin. Genet. Dev. 3 (1993), 50-54; Bates and Vousden, Curr. Opin. Genet. Dev. 6 (1996), 12-19).

Although tumor suppressor genes have recently attracted a lot of attention due to the possibility that they may provide important targets in the treatment of cancer, only a limited number of TSGs could be identified and cloned. Thus, there still exists a need for the identification of further tumor suppressor genes in order to better understand the mechanisms of the development of diseases such as cancer and to be able to provide means for the treatment of further forms of tumorous diseases or for the improved treatment of tumorous diseases. One reason for the slow progress in cloning TSGs may be seen in the fact that there exists no method for the identification and isolation which can be easily carried out in-vitro and allows the rapid screening of a plurality of potential sequences for tumor suppressor activity.

Thus, the technical problem underlying the present invention is to provide further nucleic acid molecules coding for proteins displaying tumor suppressor activity as well as methods for their identification and isolation.

The solution to the above technical problem is achieved by providing the embodiments characterized in the claims. Namely, nucleic acid molecules coding for a novel class of tumor suppressor proteins have been identified. This has been achieved by using an in-vitro functional expression transducing cloning technique. The described novel class of tumor suppressor proteins shares the ability of p53 to inhibit growth of tumor cells by controlling apoptotic cell death and cell cycle progression and appears to play a critical role in apoptosis and cell cycle regulation. However, the newly identified tumor suppressors display a more restricted pattern of tissue expression and distinct activities compared to known TSGs such as p53.

Thus, in one aspect, the present invention relates to a nucleic acid molecule encoding a protein having the biological activity of a tumor suppressor selected from the group consisting of:

- (a) nucleic acid molecules coding for a polypeptide comprising the amino acid sequence given in SEQ ID NO.2;
- (b) nucleic acid molecules comprising the nucleotide sequence given in SEQ ID NO.1;
- (c) nucleic acid molecules hybridizing to a nucleic acid molecule as defined in (a) or (b); and
- (d) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in (a), (b) or (c).

The nucleic acid molecule with the nucleotide sequence of the coding region as depicted in SEQ ID NO. 1 codes for a protein of 667 amino acids with a predicted molecular weight of 75 kDa (Figure 1A). The ATG of AGGCCATGG (SEQ ID NO.

4) was assigned as initiation codon on the basis of its close match to the CC(A/G)CCATGG (SEQ ID NO. 5) Kozak consensus sequence for favored initiation of translation and the presence of an in-frame TGA stop codon 12 nucleotides upstream. Data base searches revealed the presence of seven zinc fingers (Klug and Schwabe, FASEB J. (1995), 597-604) in the N-terminal region. However, homologies to other members of the zinc finger protein family were low (30% for the best), with the closest group being the GLI-Krüppel family of zinc finger proteins which have been implicated in normal development and tumor formation (Ruppert et al., Mol. Cell. Biol. 8 (1988), 3104-3113). In particular, the first H/C link (HSRERPFKC (SEQ ID NO. 6)) is in good agreement with the consensus motif for the GLI-Krüppel family (H(S/T)GEKP(F/Y)XC (SEQ ID NO. 7)) (Schuh et al., Cell 47 (1986), 1025-1032). On the other hand, the remaining 459 C-terminal amino acids displayed no significant homologies to sequences in the Swissprot and NBRF-PIR data bases. The central region of the protein (275-383) is characterized by 34 PLE, PMQ or PML repeats, suggestive of a structure known as poly proline type II helix which is considered to be critically involved in protein-protein interactions (Williamson, Biochem. J. 297 (1994), 249-260). The COOH-terminal region is particularly P-, Q- and E-rich, a feature often displayed by transactivation domains of transcription factors. In addition, the presence of a putative phosphorylation site (HSPQK (SEQ ID NO. 8)) for cyclin-dependent kinases (Cdks) located between the second and third zinc finger motif (residues 56-60) as well as a putative protein kinase A (PKA)-phosphorylation site (KKWTI (SEQ ID NO. 9)) at the very C-terminus (residues 663-666) suggests possible regulation by protein kinases.

Studies which had been carried out in the scope of the present invention revealed that the protein encoded by the nucleic acid sequence of SEQ ID NO. 1 displays the biological activity of a tumor suppressor.

The term "tumor suppressor", as used herein, relates to any protein/polypeptide inhibiting growth of tumor cells in-vitro and/or in-vivo. Growth inhibition involves mechanisms such as control of apoptosis and/or of cell cycle progression as well

as mechanisms unidentified so far. "Tumor suppressors" are proteins displaying biological activities identical to or similar to those of p53, Rb (retinoblastoma gene product), WT (Wilms tumor suppressor gene), VHL (von Hippel-Lindau tumor suppressor gene), BRCA1 (breast cancer susceptibility gene) and p16 (cyclin-dependent kinase inhibitor).

Examples for important biological activities of a tumor suppressor are the capability to inhibit in-vitro proliferation of tumor cells as evidenced for instance by measuring colony formation, growth rate and cloning in soft agar as well as the capability to inhibit in-vivo tumor formation in nude mice. These biological activities can be determined, for example, according to Zhou et al., Proc. Natl. Acad. Sci. USA 91 (1994), 4165-4169; Chen et al., Science 250 (1990), 1576-1580; Baker et al., Science 249 (1990), 912-915; Diller et al., Mol. Cell. Biol. 10 (1990), 5772-5781; Casey et al., Oncogene 6 (1991), 1791-1797; Cheng et al., Cancer Research (1992), 222-226; Wang and Prives, Nature 376 (1995), 88-91; Mercer et al., Proc. Natl. Acad. Sci. USA 87 (1990), 6166-6170; Antelman et al., Oncogene 10 (1995), 697-704 or as described in the appended examples.

The protein encoded by the nucleic acid sequence of SEQ ID NO. 1 displays the ability to suppress tumor cell proliferation which could be demonstrated by the constitutive and induced expression of said protein in transfected tumor cells. Furthermore, said protein is capable of inhibiting anchorage-independent growth which is often correlated with tumorigenesis and is a strong criteria for cultured cell transformation. Furthermore, this novel protein is able to suppress tumor formation of transformed cells injected in nude mice. Thus, the protein of the invention displays all essential features of a tumor suppressor similar to those of, for example, p53. This new tumor suppressor is also able to induce apoptosis resulting in inhibition of tumor cell growth. However, this new tumor suppressor exhibits functional differences compared to p53, for instance the induction of apoptotic cell death is more pronounced in Saos-2 cells for the protein of the invention than for p53. Furthermore, the tumor suppressor of the invention induces G1 arrest of the cell cycle, in contrast to p53, independently from the

transactivation of the gene encoding the cyclin-dependent kinase inhibitor p21. Finally, it had been shown that this protein acts as nuclear transcription factor.

From the above it is evident that the nucleotide sequence depicted in SEQ ID NO. 1 codes for a novel class of tumor suppressors. By the provision of this nucleotide sequence it is now possible to isolate identical or similar nucleic acid molecules which code for proteins with the biological activity of a tumor suppressor from other species or organisms. Well-established approaches for the identification and isolation of such related sequences are, for example, the isolation from genomic or cDNA libraries using the complete or part of the disclosed sequence as a probe or the amplification of corresponding nucleic acid molecules by polymerase chain reaction using specific primers.

Thus, the invention also relates to nucleic acid molecules which hybridize to the above described nucleic acid molecules and differ at one or more positions in comparison to these as long as they encode a protein having tumor suppressor activity. Such molecules comprise those which are changed, for example, by deletion(s), insertion(s), alteration(s) or any other modification known in the art in comparison to the above described nucleic acid molecules. Methods for introducing such modifications in the nucleic acid molecules according to the invention are well-known to the person skilled in the art; see, e.g., Sambrook et al. (Molecular cloning; A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, Cold Spring Harbor NY (1989)). The invention also relates to nucleic acid molecules the sequence of which differs from the sequence of any of the above-described molecules due to the degeneracy of the genetic code.

With respect to the sequences characterized under (c) above, the term "hybridizing" in this context is understood as referring to conventional hybridization conditions, preferably such as hybridization in 50%formamide/6xSSC/0.1%SDS/100µg/ml ssDNA, in which temperatures for hybridization are above 37°C and temperatures for washing in 0.1xSSC/0.1%SDS

are above 55°C. Most preferably, the term "hybridizing" refers to stringent hybridization conditions, for example such as described in Sambrook, supra.

Nucleic acid molecules which hybridize to molecules according to the invention and encode a tumor suppressor may, for example, be those which code for proteins in which putative phosphorylation sites are altered. Biochemical analysis of the regulation of wild-type p53 sequence-specific DNA binding has, for instance, shown that the unphosphorylated tetramer has a cryptic sequence-specific DNA binding activity. This cryptic or latent state of p53 depends upon a C-terminal negative regulatory domain, which locks the unphosphorylated tetramer in an inactive state. Phosphorylation of the C-terminal negative regulatory domain of latent p53 by either protein kinase C or casein kinase II or deletion of the regulatory domain activates sequence-specific DNA binding. In addition, a monoclonal antibody can mimic the effects of protein kinases and activate latent p53. Thus, neutralization of this negative regulatory domain by covalent or non-covalent modification is an important stage in the activation of p53. As described above, the protein encoded by SEQ ID NO. 1 has two putative phosphorylation sites for protein kinases. A consensus motive for cyclin-dependent kinases is located in the DNA-binding domain raising the possibility that the DNA-binding affinity can be finetuned by the driving forces of the cell cycle, constituting a putative feedback loop. This regulatory site could offer the interesting possibility that molecular tools directed against cyclin/cyclin-dependent kinase can via this loop modify the activity of this protein and implement apoptosis proficiency to target tissues. Similarly the presence of a motif for protein kinase A at the very C-terminus of the protein transactivations domain could be a critical target to modulate transactivation potency.

The above-described nucleic acid molecules which encode a novel class of tumor suppressors had been identified by using an in-vitro functional transducing cloning technique. Thus, in another aspect, the present invention relates to a process for the identification and cloning of nucleic acid molecules encoding a

protein having the biological activity of a tumor suppressor comprising the steps of:

- (i) transfecting mammalian cells with
 - (a) a first vector comprising a scorable reporter gene operatively linked to regulatory elements comprising at least one cAMP responsive element so located relative to said reporter gene to permit cAMP inducible expression thereof; and
 - (b) pools of expression vectors comprising nucleic acid molecules linked to regulatory elements allowing expression in the mammalian cells;
- (ii) cultivating the transfected cells under conditions which permit expression of the nucleic acid molecules present in the vectors;
- (iii) identifying those vector pools which lead after transfection to expression of said reporter gene in the mammalian cells;
- (iv) optionally subdividing the vector pool(s) identified in step (iii) and repeating step (i) to (iii); and
- (v) isolating from the so-identified vector pool(s) the nucleic acid molecule present in the vector(s) and testing its product for tumor suppressor activity.

This novel functional expression cloning technique relies on the transcriptional induction of a gene coding for a G-protein coupled receptor (GPCR) which in its activated form stimulates the cAMP signaling pathway which in turn results in the induction of cAMP responsive genes.

In the method of the invention said transcriptional induction of GPCR genes is conferred by the expression of a functional tumor suppressor, the presence of which is detected by subsequent activation of the endogenous signal transduction pathway and can be monitored by activation of a downstream amplifier, for example, a cAMP responsive reporter gene. The GPCR the expression of which is induced by the tumor suppressor may be any GPCR which is active under the culture conditions employed, in the sense that it activates the cAMP signaling

pathway. For example, the GPCR may be constitutively active or activated by a cognated ligand. Examples for GPCR which are positively coupled to cAMP production are the calcitonin, parathyroid hormone, thyrotropin, β -adrenergic and pituitary adenylate cyclase activating peptide (PACAP) receptors. One may specifically target a tumor suppressor which induces the transcription of a certain GPCR by adding a ligand or a certain combination of different ligands which activate the cognate GPCR the expression of which is induced by the tumor suppressor. In a preferred embodiment the ligand is the peptide PACAP and the GPCR is the PACAP-type 1 (PVR1) receptor (Spengler et al., Nature 365 (1993), 170-175).

As mentioned above, the GPCR may not require to be activated by the addition of a ligand. These may be a, for example, naturally occurring constitutive active native or mutated GPCR.

Regulation of PVR1-receptor expression depends on activation of the endogenous gene by mechanisms at present not known. Delineation of this molecular pathway could allow to determine the cis-regulatory sequences in the PVR-1 receptor used for transactivation by TSGs. Therefore fusion of such a TSG-responsive region to a reporter gene could present an alternative usage of the present method.

Cells which are suitable for the purpose of the described method are such cells which reveal to elevations of intracellular cAMP a nuclear response leading to transcriptional activation of genes linked to a cAMP-responsive element.

Examples for such cells are those of cell line porcine renal epithelial LLC-PK1 (ATCC CC101) and human osteosarcoma Saos-2 (ATCC HTB 85). A suitable cell line is characterized by the presence of a cAMP dependent protein kinase A (PKA) and a cAMP response element (CRE)-binding protein which mediate the effects of cAMP. After binding of cAMP PKA is activated and able to phosphorylate the CRE-binding protein which is activated to turn on the transcription of cAMP responsive genes, namely genes which contain a short regulatory sequence called CRE which provide for binding of the CRE-binding

protein; for a general review of the cAMP signaling pathway; see, for example, Alberts et al., *Molecular Biology of the Cell*, 3rd ed. Garland Publ., Inc. N.Y. (1994) Chapter 15.

Other suitable cell lines may be identified by the person skilled in the art by screening a panel of cell lines for efficient expression of the expression vector employed combined with high transfection efficiency and with high responsiveness to cAMP. Expression and transfection efficiency may be optimized by conventional methods known in the art. Responsiveness to cAMP can be determined, for example, by transfection of a plasmid encoding and expressing a receptor which is positively coupled to cAMP production such as, for example, a GPCR as described above and measuring the induction of the cAMP mediated cellular response. The cAMP mediated cellular response may be determined by, for example, quantifying the production of cAMP or by monitoring the activation of an endogenous cAMP responsive gene and/or of a cotransfected cAMP responsive reporter gene. A detailed method for identifying a suitable cell line is described, for instance, in Example 1.

The cAMP-responsive element present in the regulatory elements which drive expression of the reporter gene on the first vector may in principle be any element known to respond to elevated levels of intracellular cAMP with an increase of transcription rate of a cis-linked sequence. Such cAMP-responsive elements are known, for example, from the genes encoding peptide hormones, for example somatostatin and corticotropin releasing hormone and are described in Spengler et al., *Mol. Endocrinology* 6 (1992), 1931-1941; Comb et al., *Nature* 323 (1986), 353-356; Roesler et al., *J. Biol. Chem.* 263 (1988), 9063-9066; Karin, *Trends Genet.* 5 (1989), 65-67 and Lalli and Sassone-Corsi, *J. Biol. Chem.* 269 (1994), 17359-17362. Preferably, the cAMP-responsive element has the nucleotide sequence of the consensus sequence of cAMP-responsive elements described in the literature. Most preferably, the cAMP-responsive element is one known from a human corticotropin releasing hormone gene which is, advantageously, flanked

by sequences naturally surrounding it. Advantageous is also the use of a cAMP-responsive element comprising the consensus sequence of an AP1 element or a degenerated version thereof.

The regulatory elements comprising the cAMP-responsive element(s) and which direct expression of the reporter gene in the transfected cells, may be any suitable elements capable of directing expression in the chosen cells. These elements normally comprise a promoter sequence, in particular a minimal promoter, preferably one which comprises

- (a) a TATA or a CAAT box, preferably in conjunction with an Sp1-dependent activator, or
- (b) an initiator element (Inr) in conjunction with an Sp1-dependent activator.

In a preferred embodiment the regulatory elements are derived from mammary mouse tumor virus (MMTV) promoter.

The reporter gene present in the first vector may be any suitable reporter gene the expression of which can be detected in the transfected cells. Preferably, a reporter gene is chosen the expression of which can be easily detected, for example, by photometric or fluorometric methods, isotopic labeling or by a staining reaction. Examples for reporter genes preferably used in the method according to the invention are those coding for chloramphenicol-acetyltransferase (CAT), β -galactosidase (β -Gal), secreted alkaline phosphatase (SEAP) or growth hormone (GH). Most preferably, a gene coding for luciferase is used.

The term "pool of expression vectors" in step (i) (b) of the method according to the invention is meant to be understood as a plurality of vector molecules which are either identical or not and which are adapted for expression in the transfected cells. Such vector molecules comprise regulatory elements which are capable of directing expression of a linked sequence in the transfected cells. Furthermore, these vector molecules comprise nucleic acid sequences linked to said regulatory elements which code for a gene product and which may either be identical or different in the members of the vector pool. The vectors of said vector pool may

furthermore comprise sequences which ensure replication in prokaryotic host cells as well as sequences which ensure replication in the transfected eukaryotic cells. Such a pool of expression vectors may be, for example, a cDNA library or a genomic library cloned in expression vectors suitable for expression in the transfected cells.

In this context, suitable expression vectors are known in the art such as Okayama-Berg cDNA expression vector pcDV1 (Pharmacia), pCDM8, pRc/CMV, pcDNA1, pcDNA3 (In-vitro gene), pSPORT1 (GIBCO BRL).

In a preferred embodiment the nucleic acid molecules present in the vectors of the vector pool are cDNA molecules. In particular, said cDNA may be prepared from RNA obtained from any organism or tissue, namely from any animal, bacterial, fungal or plant cells or from viruses. Most preferably, the RNA is obtained from mammalian cells. In such case the RNA is preferably derived from a specific tissue or organ of a mammal, e.g., intestine, stomach, lung, adrenal gland, kidney, mammary gland, prostate, testis, most preferably said tissue is pituitary gland, brain or ovary.

If a vector pool is identified in step (iii) of the method according to the invention then it is either possible to isolate from the original pool of the so-identified vector pool the nucleic acid molecules present in the vectors of the vector pool and characterize the encoded products or one can further subdivide the original vector pool, for example, if it consists of vectors with a plurality of different inserts, so as to reduce the number of different vectors per pool and repeat the method with the subdivisions of the original pool. Depending on the complexity of the pool this can be done for several times, preferably so often until the vector pool identified in step (iii) of the method only comprises a limited number of vectors which differ with respect to their inserted nucleic acid molecule. Normally the vector pool used in step (i) for transfecting the cells has previously been isolated from a mixture of bacteria harboring different vectors and which, thus, constitute a kind of library. Subdivision of the vector pool for the purpose of step (iii) of the method can

therefore be achieved by subdividing said library comprising the bacteria so that the diversity of the vectors with respect to the inserted nucleic acid sequences is lower in the subdivisions than in the original library. From these subdivisions of the library the expression vectors can then be isolated. These isolates then represent subdivisions of the original vector pool.

The nucleic acid molecules present in vectors of a vector pool identified by (iii) of the method according to the invention can be isolated from the vectors, e.g. by digestion with suitable restriction enzymes and can be further characterized, for example by restriction mapping, sequencing etc. The expression products of the thus obtained nucleic acid molecules are then tested for their tumor suppressor activity. This can be done, for example, by measuring the suppression of colony formation of transformed cell lines transfected with a TSG expressing vector. Furthermore, an inducible expression system may be employed to measure the suppression of growth of tumor cells which are stably transfected with an inducible TSG. After induction of TSG expression the growth of the tumor cells can be monitored and compared to non-TSG expressing tumor cells. An example of an inducible expression system is the tetracycline-regulated gene expression but others may be used as well, for example, heavy metal inducible expression systems. The suppressor activity of the putative TSG can also be detected by assaying its ability of suppressing anchorage-independent cell growth after induction of the TSG of the stably transfected tumor cells. Furthermore, the loss of tumorigenicity under expression of the TSG can be tested, for example, by implanting the tumor cells harboring an inducible TSG in nude mice and monitoring tumor development after induction of TSG expression. The capability of TSGs of recruiting apoptotic programs to inhibit growth of tumor cells is evidenced, for example, by the failure of the induced TSG expressing cells to convert MTT, their shrinking in size, their abundance in phase contrast microscopy, blebbing of their membrane, and rounding up of the cells before detaching from the plates. The cell death may also be accompanied by fragmentation of the DNA into a ladder of regular subunits. All the methods

referred to are well known in the art and are described in the Examples of the present application and/or are described in Zhou et al., Proc. Natl. Acad. Sci. USA 91 (1994), 4165-4169; Chen et al., Science 250 (1990), 1576-1580; Baker et al., Science 249 (1990), 912-915; Diller et al., Mol. Cell. Biol. 10 (1990), 5772-5781; Casey et al., Oncogene 6 (1991), 1791-1797; Cheng et al., Cancer Research (1992), 222-226; Wang and Prives, Nature 376 (1995), 88-91; Mercer et al., Proc. Natl. Acad. Sci. USA 87 (1990), 6166-6170; Antelman et al., Oncogene 10 (1995), 697-704; Gossen et al., Trends Biotech. 12 (1994), 58-62; Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551.

The expression products of the identified nucleic acid molecules can be furthermore characterized by expressing them in prokaryotic host cells and purifying them. Subsequently, enzymatic and/or other biological activities can be determined by in-vitro assays. Expression in eukaryotic host cells or in-vitro transcription and translation systems may furthermore provide information about possible phosphorylation and/or glycosylation patterns etc.

As demonstrated in the examples of the present invention, the above-described method is suitable to identify and isolate nucleic acid molecules which encode proteins having the biological activity of a tumor suppressor.

Thus, in another aspect the present invention relates to nucleic acid molecules obtainable by a method according to the invention which encode a protein or polypeptide having tumor suppressor activity. Examples for such nucleic acid molecules are those described above. In a preferred embodiment the nucleic acid molecules according to the invention are DNA molecules, most preferably cDNA molecules.

Nucleic acid molecules according to the invention can be derived from any organism, namely from animals, plants, fungi, bacteria or viruses. In a preferred embodiment the nucleic acid molecules according to the invention are derived from a mammal, most preferably from a human or a mouse.

With the help of nucleic acid molecules identified and isolated by the method according to the invention it is possible to isolate the same or related molecules from the same or different organisms, for example, by screening genomic or

cDNA libraries with the nucleic acid molecules isolated according to described method as a probe.

Thus, the present invention also relates to nucleic acid molecules which hybridize to a nucleic acid according to the invention as described above and which code for a protein having tumor suppressor activity. In accordance with the present invention, a further nucleic acid molecule encoding a protein having tumor suppressor activity was isolated using a nucleic acid molecule comprising the coding sequence of SEQ ID NO. 1 as a probe. The nucleotide sequence of said nucleic acid molecule is given in SEQ ID NO. 16 encoding a protein having the amino acid sequence as depicted in SEQ ID NO. 17.

The overall identity between the coding sequences of SEQ ID NO. 1 and SEQ ID NO. 16 was 74.6% at the nucleotide level and 68.5% at the amino acid level. As described in Example 9, several domains could be identified in the amino acid sequence of this protein (SEQ ID NO. 17), each having its counterpart in SEQ ID NO. 2. As described in Examples 12 to 15, the protein encoded by the nucleic acid sequence of SEQ ID NO. 16 displays identical properties compared to those of the protein encoded by the nucleotide sequence of SEQ ID NO. 1 described above and, therefore, constitutes a member of the new class of TSGs established by the provision of the aforescribed nucleic acid molecules and proteins. Thus, in a preferred embodiment, the present invention relates to nucleic acid molecules, wherein the nucleic acid molecule comprises a nucleotide sequence encoding the amino acid sequence given in SEQ ID NO. 17 or comprises the nucleotide sequence given in SEQ ID NO. 16:

Furthermore, the present invention relates to nucleic acid molecules which hybridize to a nucleic acid molecule according to the invention as described above and which encode a mutated version of a polypeptide encoded by a nucleic acid molecule as described above which has lost its tumor suppressor activity.

Furthermore, the present invention relates to nucleic acid molecules which represent or comprise the complementary strand of any of the above described nucleic acid molecules or a part thereof. Such a molecule may either be a desoxyribonucleic acid or a ribonucleic acid. Such molecules comprise, for example, antisense RNA. Nucleic acid molecules according to this preferred embodiment of the invention which are complementary to a nucleic acid molecule as described above may also be used for repression of expression of a TSG, for example due to an antisense or triple helix effect or for the construction of appropriate ribozymes (see, e.g., EP-B1 0 291 533, EP-A1 0 321 201, EP-A2 0 360 257) which specifically cleave the (pre)-mRNA of a gene comprising a nucleic acid molecule of the invention. Selection of appropriate target sites and corresponding ribozymes can be done as described for example in Steinecke, Ribozymes, Methods in Cell Biology 50, Galbraith et al. eds Academic Press, Inc. (1995), 449-460.

The present invention also relates to nucleic acids molecules of at least 15 nucleotides in length which specifically hybridize to any one of the aforementioned nucleic acid molecules or to a complementary strand thereof. Said nucleic acid molecules may be used, for example, as probes for the detection of a TSG according to the invention or its mRNA. In a preferred embodiment said nucleic acid molecules are labeled. Methods for the detection of nucleic acids are well known in the art, e.g., Southern and northern blotting, PCR, primer extension. In another preferred embodiment said nucleic acid molecules may be used for the suppression of TSG expression.

Furthermore, the present invention relates to a vector comprising a nucleic acid molecule according to the invention. Examples for such vectors are pUC18, pBR322, pBlueScript.

In a preferred embodiment the nucleic acid molecule present in the vector is operatively linked to regulatory elements permitting expression in prokaryotic or

eukaryotic host cells. Possible regulatory elements permitting expression in prokaryotic host cells comprise, e.g., the *lac* or *trp* promoter in *E. coli*, and examples for regulatory elements permitting expression in eukaryotic host cells are the *AOX1* or *GAL1* promoter in yeast or the CMV-, SV40-, RSV-promoter (Rous sarcoma virus), CMV-enhancer, SV40-enhancer or a globin intron in mammalian and other animal cells. Beside elements which are responsible for the initiation of transcription such regulatory elements may also comprise transcription termination signals, such as the SV40-poly-A site or the tk-poly-A site, downstream of the nucleic acid molecule.

The invention also relates to a host cell comprising a vector according to the invention. In this context, the host cell may be a bacterial, fungal, plant or animal cell. In a preferred embodiment the host cell is a mammalian cell.

In a further embodiment the invention relates to a method for the production of a polypeptide having the biological activity of a tumor suppressor comprising culturing a host cell as defined above under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture. Such methods are described, for example, in LaVallie and McCoy, Curr. Opin. Biotech. 6 (1995), 501-506; Wong, Curr. Opin. Biotech. 6 (1995), 517-522; Romanos, Curr. Opin. Biotech. 6 (1995), 527-533; Keränen and Penttilä, Curr. Opin. Biotech. 6 (1995), 534-537; Williams et al., Curr. Opin. Biotech. 6 (1995), 538-542; Davies, Curr. Opin. Biotech. 6 (1995), 543-547; Holmgren, Annu. Rev. Biochem. 54 (1985) 237-271 or LaVallie et al., Bio/Technology 11 (1993) 187-193.

Furthermore, the invention relates to a polypeptide encoded by a nucleic acid molecule according to the invention or produced by the above-described method, which has tumor suppressor activity.

In this context it is also understood that the polypeptides according to the invention may be further modified by conventional methods known in the art. By providing the polypeptides according to the present invention it is also possible to determine the portions relevant for their biological activity, namely their tumor suppressor activity. This may allow the construction of chimeric proteins comprising an amino acid sequence derived from a tumor suppressor protein of the invention which is crucial for tumor suppression and other functional amino acid sequences e.g. nuclear localization signals, transactivating domains, DNA-binding domains, hormone-binding domains, protein tags (GST, GFP, h-myc peptide, Flag, HA peptide) which may be derived from the same or from heterologous proteins.

The present invention also relates to a polypeptide encoded by a nucleic acid molecule according to the invention or produced by the above-described method, which is a mutated version of an above-described polypeptide which has lost its tumor suppressor activity.

The present invention furthermore relates to antibodies specifically recognizing a polypeptide according to the invention which has a tumor suppressor activity. Namely, the invention relates to antibodies which specifically recognize polypeptides according to the invention irrespective of whether they are functional tumor suppressors or whether they are mutated forms which have lost their tumor suppressor activity.

In a preferred embodiment the antibody specifically recognizes a polypeptide according to the invention which has tumor suppressor activity but does not recognize a polypeptide which is a mutated version of such a polypeptide and which has lost its tumor suppressor activity.

In another preferred embodiment the antibody specifically recognizes the mutated form which has lost its tumor suppressor activity but not the corresponding polypeptide having tumor suppressor activity.

In a preferred embodiment said antibody is a monoclonal antibody.

Anti-tumor suppressor protein antibodies can be prepared by well known methods using a purified tumor suppressor protein according to the invention or a synthetic fragment derived therefrom as an antigen. Monoclonal antibodies can be prepared, for example, by the techniques as described in Köhler and Milstein, Nature 256 (1975), 495, and Galfré, Meth. Enzymol. 73 (1981), 3, which comprise the fusion of mouse myeloma cells to spleen cells derived from immunized mammals. The antibodies can be monoclonal antibodies, polyclonal antibodies or synthetic antibodies as well as fragments of antibodies, such as Fab, Fv or scFv fragments etc. Furthermore, antibodies or fragments thereof to the aforementioned tumor suppressor proteins can be obtained by using methods which are described, e.g., in Harlow and Lane "Antibodies, A Laboratory Manual", CSH Press, Cold Spring Harbor, 1988. These antibodies can be used, for example, for the immunoprecipitation and immunolocalization of the proteins of the invention as well as for the monitoring of the presence of such proteins, for example, in recombinant organisms, and for the identification of compounds interacting with the proteins according to the invention. For example, surface plasmon resonance as employed in the BIAcore system can be used to increase the efficiency of phage antibodies which bind to an epitope of the protein of the invention (Schier, Human Antibodies Hybridomas 7 (1996), 97-105; Malmberg, J. Immunol. Methods 183 (1995), 7-13).

Moreover, the present invention relates to a pharmaceutical composition comprising at least one of the aforementioned nucleic acid molecules, vectors, polypeptides and/or antibodies according to the invention either alone or in combination, and optionally a pharmaceutically acceptable carrier or excipient.

Examples of suitable pharmaceutical carriers are well known in the art and include phosphate buffered saline solutions, water, emulsions, such as oil/water emulsions, various types of wetting agents, sterile solutions etc. Compositions comprising such carriers can be formulated by conventional methods. The pharmaceutical compositions can be administered to the subject at a suitable dose. Administration of the suitable compositions may be effected by different

ways, e.g. by intravenous, intraperitoneal, subcutaneous, intramuscular, topical or intradermal administration. The dosage regimen will be determined by the attending physician and other clinical factors. As is well known in the medical arts, dosages for any one patient depends upon many factors, including the patient's size, body surface area, age, the particular compound to be administered, sex, time and route of administration, general health, and other drugs being administered concurrently. Generally, the regimen as a regular administration of the pharmaceutical composition should be in the range of 1 μ g to 10 mg units per day. If the regimen is a continuous infusion, it should also be in the range of 1 μ g to 10 mg units per kilogram of body weight per minute, respectively. Progress can be monitored by periodic assessment. Dosages will vary but a preferred dosage for intravenous administration of DNA is from approximately 10^6 to 10^{22} copies of the DNA molecule. The compositions of the invention may be administered locally or systemically. Administration will generally be parenterally, e.g., intravenously; DNA may also be administered directly to the target site, e.g., by biolistic delivery to an internal or external target site or by catheter to a site in an artery.

The pharmaceutical compositions according to the invention can be used for the prevention or treatment of different kinds of diseases, for example, cancer, namely benign or malignant tumors, of acquired or inborn neuronal disorders, neurodegenerations and related disorders. With respect to the prevention or treatment of tumors, said tumors are preferably derived from endocrine or neuronal tissues, i.e. intestine, stomach, lung, adrenal gland, kidney, mammary gland, prostate, testis, most preferably said tissue is colon, pancreas, thyroid, pituitary gland, brain, breast or ovary.

In a first aspect it is possible to use a pharmaceutical composition which comprises a nucleotide sequence which encodes a non-mutated form of a protein having tumor suppressor activity for gene therapy. As described above tumors or other diseases often evolve when cells lose both functional copies of a tumor suppressor gene. In such a case introduction of functional copies of the

corresponding gene may help to ameliorate the situation. For example, research pertaining to gene transfer into cells of the nervous system is one of the fastest growing fields in neuroscience. Gene therapy, which is based on introducing therapeutic genes into cells of the nervous system by ex-vivo or in-vivo techniques is one of the most important applications of gene transfer. Gene therapy of the nervous system could be applied for three general purposes: genetic diseases, acquired degenerative encephalopathies, and control of malignant neoplasia.

In genetic diseases the introduction of a normal or a functionally adequate allele of a mutated nuclear gene represents gene-replacement therapy, which is applicable mainly to monogenetic recessive disorders.

In the field of neurobiology, for example, it has long been recognized that the balance between cellular proliferation and cell death during embryogenesis is a key factor in formation of the central nervous system (CNS). The recent definition of molecular mechanisms that drive the cell-division cycle and programmed cell death provides an opportunity to investigate the molecular interactions that coordinate cell-cycle regulation with CNS-pattern formation, neural differentiation and histogenesis. It appears evident that not only is the cell-division cycle regulated by developmentally controlled molecular signals to halt or proceed, but gene products that drive the cycle can also influence the course of neural differentiation and apoptosis. The neurotrophic strategy for the regulation of neuronal numbers may be only one example of a general mechanism that help to regulate the numbers of many other vertebrate cell types, which also require signals from other cells to survive. These survival signals seem to act by suppressing an intrinsic cell suicide program, the protein components of which are apparently expressed constitutively in most cell types. TSGs have emerged during the last years as major players in this area. Mice deficient for Rb revealed massive neuronal cell death due to the failure to stop cell division. A subset of p53-deficient mice (8 to 16%) exhibit exencephaly and a large population (40%) of Brca1-deficient mice embryos suffered to varying degrees of spina bifida and anencephalopathy. In addition, these animals displayed a disorganisation of the

neuroepithelium with signs of rapid proliferation and excessive cell death. Thus, it appears as if TSGs are intimately involved in CNS formation and that the balance between growth-constraining elements and neurotrophic support is a key event in formation of neuronal architecture. In this respect, nucleic acid molecules according to the invention which code for proteins with tumor suppressor activity are potential candidates to participate in these processes. Importantly, the nucleotide sequences of SEQ ID NO. 1 and SEQ ID NO. 16 which encode novel tumor suppressors hybridize to total RNA isolated from different brain areas of the mature brain. This opens the perspective that the subtle balance between promoters of apoptosis like the protein encoded by SEQ ID NO. 1 or SEQ ID NO. 16 and protectors like neurotrophins safeguard functional integrity of the mature brain. An increasing list of neurodegenerative disorders including Alzheimer disease and Chorea Huntington have been reported to reveal increased incidence of apoptotic cell death. In this view gene targeting of nucleic acid molecules coding for a protein having the amino acid sequence as depicted in SEQ ID NO. 2 or SEQ ID NO. 16 or nucleic acid molecules coding for related proteins of the invention bears the potential promise to mitigate apoptotic cell death under various circumstances and to increase sensitivity to neurotrophic treatments aimed to preserve neuronal cell number and neuronal viability.

Furthermore, recent reports indicated that p53-dependent apoptosis modulates the cytotoxic effects of common antitumor agents such as ionizing radiation, fluorouracil, etoposide, and doxorubicin. Cells lacking wild-type p53 are resistant to these agents, whereas cells expressing wild-type p53 are sensitive to them and undergo cell death by apoptosis. These observations raise the exciting prospect that p53 mutations may provide a genetic basis for drug resistance. In the presence of p53, oncogene-expressing cells can form tumors, but cell survival is limited by their increased susceptibility to apoptosis. Conversely, p53 loss contributes directly to immortalization and tumorigenesis, probably by abrogating an intrinsic senescence program. As a consequence, selection against p53 often occurs late in tumor progression. Anticancer agents may simply activate the apoptotic program intrinsic to these sensitized cells. These observations predict

that reintroduction of normal tumor suppressor function into tumors harboring mutations in tumor suppressor genes will enhance apoptosis after radiation or chemotherapy, an approach that has proved successful for cisplatin in a lung carcinoma cell line.

Since it was observed that introduction of a nucleic acid molecule according to the invention caused apoptotic cell death in transformed cell lines, which in part exceeded the one caused by p53, these novel TSGs present a powerful option of high potential interest in gene therapy experiments. Though p53 and the protein encoded by SEQ ID NO. 1 or SEQ ID NO. 16 induce at a descriptive level the same responses, namely cell-cycle regulation and cell death, the underlying molecular routes diverge. This observation originates from the fact that the DNA-binding domain of the protein encoded by SEQ ID NO. 1 and SEQ ID NO. 16 is organized in a typical zinc finger structure, which is unrelated to the central DNA-binding domain of p53. Therefore, the protein encoded by SEQ ID NO. 1 and SEQ ID NO. 16 and related proteins could replace p53 in gene therapy strategies. Importantly p53 seems only to trigger growth arrest and not cell death in some cell types and under some conditions. In line with this view we demonstrated that restoration of inducible p53 function in the p53-negative cell line Saos-2 (human, osteosarcoma) installed preferentially a growth and a comparatively weak apoptotic response, whereas Saos-2 cells became highly apoptosis proficient under expression of the protein encoded by SEQ ID NO. 1 and SEQ ID NO. 16. This differential apoptotic response emphasizes the idea that this protein and other TSGs of the invention and p53 supply different molecular routes to apoptosis and open the exciting perspective that apoptosis competency is a tissue-specific encoded genetic program. Conclusively tissue-specific TSGs as those provided by the present invention could encode specific properties to guide tumorigenic cells to apoptotic cell death and their potency to do so could surpass p53 as illustrated for the proteins encoded by SEQ ID NO. 1 and SEQ ID NO. 16 in Saos-2 cells.

Importantly again, the understanding of p53 function as an example for a tumor suppressor gene suggest a basis for the association between p53 mutations and

poor patient prognosis. Thus, p53 mutations, which are with 50% among the most common alterations observed in human cancer, may be a significant impediment to successful cancer therapy. For example, p53 mutations dramatically reduce the probability that patients with B cell chronic lymphocyte leukemia will enter remission after chemotherapy. Similarly evaluation of the status of proteins encoded by nucleic acid molecules according to the invention and related proteins in tumor samples could serve as an decisive parameter for the extent and necessity of surgical resection and the need for adjuvant therapy. In a more general view, the status of nucleic acid molecules according to the invention encoding proteins with the capability to induce apoptosis could become a decisive criteria to develop treatment priorities for individual tumor specisms. In another important aspect the above-mentioned pharmaceutical compositions may be used in immunotherapy. The well-characterized mutations of a TSG also suggest the possibility of immunotherapy or even a cancer vaccine, which would alert the body's immune system to the mutant forms of the protein. Cross-reactivity to wild-type forms has to be considered as a potential unwanted side-effect with profound implications since abating wild-type function through uncontrolled autoimmunoreactivity would dramatically enhance the risk of additional tumor formation. In this regard, it is advantageous to use tissue specific TSGs, such as the one represented in SEQ ID NO. 1 or in SEQ ID NO: 16, since in this way the risk of the above-mentioned unwanted side-effect can be substantially lowered. Vectors comprising a nucleic acid molecule of the invention may be stably integrated into the genome of the cell or may be maintained in a form extrachromosomally, see, e.g., Calos, Trends Genet. 12 (1996), 463-466. On the other hand, viral vectors described in the prior art may be used for transfecting certain cells, tissues or organs. Suitable gene delivery systems may include liposomes, receptor-mediated delivery systems, naked DNA, and viral vectors such as herpes viruses, retroviruses, adenoviruses, and adeno-associated viruses, among others. Delivery of nucleic acid molecules to a specific site in the body for gene therapy may also be accomplished using a biolistic delivery system,

such as that described by Williams (Proc. Natl. Acad. Sci. USA 88 (1991), 2726-2729).

Standard methods for transfecting cells with nucleic acid molecules are well known to those skilled in the art of molecular biology, see, e.g., WO 94/29469. Gene therapy to prevent or decrease the development of tumors may be carried out by directly administering the nucleic acid molecule of the invention encoding a TSG to a patient or by transfecting cells with said nucleic acid molecule of the invention *ex vivo* and infusing the transfected cells into the patient. Furthermore, research pertaining to gene transfer into cells of the germ line is one of the fastest growing fields in reproductive biology. Gene therapy, which is based on introducing therapeutic genes into cells by *ex-vivo* or *in-vivo* techniques is one of the most important applications of gene transfer. Suitable vectors and methods for *in-vitro* or *in-vivo* gene therapy are described in the literature and are known to the person skilled in the art; see, e.g., WO94/29469, WO 97/00957 or Schaper, Current Opinion in Biotechnology 7 (1996), 635-640, and references cited therein. The nucleic acid molecules comprised in the pharmaceutical composition of the invention may be designed for direct introduction or for introduction via liposomes, or viral vectors (e.g. adenoviral, retroviral) containing said nucleic acid molecule into the cell. Preferably, said cell is a germ line cell, embryonic cell, or egg cell or derived therefrom if the production of transgenic non-human animals is envisaged.

It is to be understood that the introduced nucleic acid molecules encoding the protein having the biological activity of a tumor suppressor express said protein or activator after introduction into said cell and preferably remain in this status during the lifetime of said cell. For example, cell lines which stably express said protein having the biological activity of a tumor suppressor may be engineered according to methods well known to those skilled in the art. Rather than using expression vectors which contain viral origins of replication, host cells can be transformed with the recombinant DNA molecule or vector of the invention and a selectable marker, either on the same or separate vectors. Following the introduction of

foreign DNA, engineered cells may be allowed to grow for 1-2 days in an enriched media, and then are switched to a selective media. The selectable marker in the recombinant plasmid confers resistance to the selection and allows for the selection of cells having stably integrated the plasmid into their chromosomes and grow to form foci which in turn can be cloned and expanded into cell lines. This method may advantageously be used to engineer cell lines which express the protein having the biological activity of a tumor suppressor.

A number of selection systems may be used, including but not limited to the herpes simplex virus thymidine kinase (Wigler, Cell 11(1977), 223), hypoxanthine-guanine phosphoribosyltransferase (Szybalska, Proc. Natl. Acad. Sci. USA 48 (1962), 2026), and adenine phosphoribosyltransferase (Lowy, Cell 22 (1980), 817) in tk, hgprt or aprt cells, respectively. Also, antimetabolite resistance can be used as the basis of selection for dhfr, which confers resistance to methotrexate (Wigler, Proc. Natl. Acad. Sci. USA 77 (1980), 3567; O'Hare, Proc. Natl. Acad. Sci. USA 78 (1981), 1527), gpt, which confers resistance to mycophenolic acid (Mulligan, Proc. Natl. Acad. Sci. USA 78 (1981), 2072); neo, which confers resistance to the aminoglycoside G-418 (Colberre-Garapin, J. Mol. Biol. 150 (1981), 1); hygromycin, which confers resistance to hygromycin (Santerre, Gene 30 (1984), 147); or puromycin (pat, puromycin N-acetyl transferase). Additional selectable genes have been described, for example, trpB, which allows cells to utilize indole in place of tryptophan; hisD, which allows cells to utilize histinol in place of histidine (Hartman, Proc. Natl. Acad. Sci. USA 85 (1988), 8047); and ODC (ornithine decarboxylase) which confers resistance to the ornithine decarboxylase inhibitor, 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987, In: Current Communications in Molecular Biology, Cold Spring Harbor Laboratory ed.).

In another aspect it is possible that the pharmaceutical compositions comprise the functional proteins encoded by the nucleic acid molecules according to the invention or proteins which represent mutated versions of these proteins which occur in various diseases. These compositions may either be useful to restore

normal tumor suppressor activity in cells which have lost both functional copies of the relevant gene or for immunotherapy as already described above.

Furthermore, the use of pharmaceutical compositions which comprise antisense-oligonucleotides which specifically hybridize to RNA encoding mutated versions of a tumor suppressor according to the invention or which comprise antibodies specifically recognizing such mutated versions but not the functional wild-type form is conceivable in cases in which the concentration of the mutated form in the cells should be reduced. The pharmaceutical compositions according to the invention can be used for the treatment of various kinds of diseases. Thus, the present invention also relates to methods for the treatment or prevention of tumors or neuronal disorders or for the delay of the reoccurrence of tumors or neuronal disorders which comprises the administration of an effective dose of a pharmaceutical composition according to the invention to the subject.

Furthermore, any of the aforementioned nucleic acid molecules, vectors, polypeptides and/or antibodies according to the invention either alone or in combination can be used for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject. Preferably, said disease is a tumor or a neuronal disorder, for example, a tumor or a neuronal disorder as described above.

The invention also relates to a diagnostic composition comprising at least one of the aforementioned nucleic acid molecules, vectors, polypeptides and/or antibodies according to the invention either alone or in combination, and optionally suitable means for detection.

Said diagnostic compositions may be used for methods for detecting expression of a tumor suppressor by detecting the presence of mRNA coding for a tumor suppressor which comprises obtaining mRNA from a cell and contacting the mRNA so obtained with a probe comprising a nucleic acid molecule of at least 15 nucleotides capable of specifically hybridizing with a nucleic acid molecule

encoding a tumor suppressor under hybridizing conditions, detecting the presence of mRNA hybridized to the probe, and thereby detecting the expression of the tumor suppressor by the cell.

Furthermore, the invention comprises methods of detecting the presence of a tumor suppressor of the present invention in a sample, for example, a cell sample, which comprises obtaining a cell sample from the subject, contacting said sample with one of the aforementioned antibodies under conditions permitting binding of the antibody to the tumor suppressor, detecting the presence of the antibody so bound, for example, using immuno assay techniques, for example, radioimmunoassay or enzymeimmunoassay. Furthermore, one may specifically detect and distinguish polypeptides which are functional tumor suppressors from mutated forms which have lost or altered their tumor suppressor activity by using an antibody which either specifically recognizes a polypeptide which has tumor suppressor activity but does not recognize an inactive form thereof or which specifically recognizes an inactive form but not the corresponding polypeptide having tumor suppressor activity. The antibodies of the present invention may also be used in affinity chromatography for purifying the polypeptides of the present invention and isolating them from various sources.

The invention also relates to a method for diagnosing in a subject a predisposition to a tumor or a disorder associated with the expression of a tumor suppressor allele which comprises isolating DNA from victims of the tumor or the disorder associated with the expression of a tumor suppressor; digesting the isolated DNA with at least one restriction enzyme; electrophoretically separating the resulting DNA fragments on a sizing gel; contacting the resulting gel with a nucleic acid probe as described above capable of specifically hybridizing to DNA encoding a tumor suppressor and labeled with a detectable marker; detecting labeled bands on the gel which have hybridized to the labeled probe to create a band pattern specific to the DNA of victims of the tumor or the disorder associated with the expression of a tumor suppressor; preparing the subject's DNA according to the above-mentioned steps to produce detectable labeled bands on a gel; and comparing the band pattern specific to the DNA of victims of the tumor or the

disorder associated with the expression of a tumor suppressor and the subject's DNA to determine whether the patterns are the same or different and to diagnose thereby predisposition to the tumor or the disorder if the patterns are the same. The detectable markers of the present invention may be labeled with commonly employed radioactive labels, such as, for example, ^{32}P and ^{35}S , although other labels such as biotin or mercury may be employed as well. Various methods well-known to the person skilled in the art may be used to label the detectable markers. For example, DNA sequences and RNA sequences may be labeled with ^{32}P or ^{35}S using the random primer method. Once a suitable detectable marker has been obtained, various methods well-known to the person skilled in the art may be employed for contacting the detectable marker with the sample of interest. For example, DNA-DNA, RNA-RNA and DNA-RNA hybridizations may be performed using standard procedures. Various methods for the detection of nucleic acids are well-known in the art, e.g., Southern and northern blotting, PCR, primer extension and the like. Furthermore, the mRNA, cRNA, cDNA or genomic DNA obtained from the subject may be sequenced to identify mutations which may be characteristic fingerprints of TSG mutations in tumors or disorders associated with the expression of TSG or mutated versions thereof. The present invention further comprises methods wherein such a fingerprint may be generated by RFLPs of DNA or RNA obtained from the subject, optionally the DNA or RNA may be amplified prior to analysis, the methods of which are well known in the art. RNA fingerprints may be performed by, for example, digesting an RNA sample obtained from the subject with a suitable RNA-Enzyme, for example RNase T_1 , RNase T_2 or the like or a ribozyme and, for example, electrophoretically separating and detecting the RNA fragments as described above.

It is furthermore possible to use the TSGs and proteins according to the invention for the design of "killer genes " (Da Costa et al., Proc. Natl. Acad. Sci. USA 93 (1996), 4192-4196). It has become clear that tumorigenesis is driven by alterations in genes that control cell growth and cell death. Gene therapy could be aimed at specifically kill tumor cells expressing mutated forms of tumor

suppressor genes. In outline, the target protein, i.e. the mutated tumor suppressor, binds to exogenously introduced gene products, resulting in transcriptional activation of a toxic gene. This strategy may be generally applicable to neoplastic disease in which the underlying patterns of genetic alterations or abnormal gene expression are known (Da Costa et al., Proc. Natl. Acad. Sci. USA 93 (1996), 4192-4196).

Conceivable is also the restoration of the wild-type conformation of mutated tumor suppressor proteins.

Some genetic changes lead to altered protein conformational states. For example, mutant p53 proteins possess a tertiary structure that renders them far less capable of binding to their wild-type DNA recognition elements. Restoring the normal or regulated conformation of mutated proteins is the most elegant and specific means to correct these molecular defects, although it is difficult. Of particular interest in this regard is the zinc finger structure of the protein encoded by SEQ ID NO. 1 or SEQ ID NO. 16 if the DNA-binding potency is reduced in mutated proteins. The fact that the nucleic acid molecules having the nucleotide sequence as depicted in SEQ ID NO. 1 or SEQ ID NO. 16 are expressed in a tissue-specific manner deserves particular attention. All pharmacological manipulations aimed at restoration of wild-type conformation p53, bear the risk to interfere with the wild-type function of this tumor suppressor in neighboring non-tumorigenic tissues with profound side-effects. In contrast the targeting of tissue-specific TSGs could remarkably extend the applicability of a targeting approach, since considerable higher concentrations of the molecules and/or long-lasting derivatives can be employed at a lowered risks for demetrial side-effects.

Thus, the nucleic acid molecules and encoded proteins of the present invention may also be used to design and/or identify molecules and compounds which are capable of activating or inhibiting the wild-type function of a tumor suppressor. These molecules and compounds may be small organic compounds, antibodies, peptidomimetics, PNAs or peptides (Milner, Nature Medicine 1 (1995), 879-880; Hupp et al., Cell 83 (1995), 237-245; Gibbs and Oliff, Cell 79 (1994), 193-198).

Thus, the present invention further relates to a process for identifying compounds effective as antagonists or agonists to a tumor suppressor comprising:

- (a) contacting a cell which expresses the polypeptide of the invention with a compound to be screened; and
- (b) determining if the compound inhibits or enhances activation of the tumor suppressor.

Said compounds may be comprised in, for example, samples, cell extracts from, e.g. plants, animals or microorganisms. Furthermore, said compounds may be known in the art but hitherto not known to be an antagonist/inhibitor or agonist/activator of the protein of the invention. Preferably said sample comprises substances of similar chemical and/or physical properties, most preferably said substances are identical. The compounds which can be prepared and identified according to a use of the present invention may be expression libraries, e.g., cDNA expression libraries, peptides, proteins, nucleic acids, antibodies, small organic compounds, ligands, hormones, peptidomimetics, PNAs or the like. The identification of compounds which are capable of activating or inhibiting the wild-type function of a tumor suppressor can be performed according to the methods known in the art, for example as described in EP-A-0 403 506. The antagonists identified according to the above-described method may reveal new classes of substances involved in, e.g., tumorigenesis. The agonist identified according to the method of the invention may prove useful for therapy of tumorous diseases. Hence, the present invention also relates to antagonists/inhibitors and agonists/activators to the polypeptide of the invention or obtainable according to the method described above.

These and other embodiments are disclosed or are obvious from and encompassed by the description and examples of the present invention. For example, further literature concerning any one of the methods, uses and compounds to be employed in accordance with the present invention may be retrieved from public libraries, using for example electronic devices. For example the public database "Medline" may be utilized which is available on Internet, e.g.

under <http://www.ncbi.nlm.nih.gov/PubMed/medline.html>. Further databases and addresses, such as <http://www.ncbi.nlm.nih.gov/>, <http://www.infobiogen.fr/>, http://www.fmi.ch/biology/research_tools.html, <http://www.tigr.org/>, are known to the person skilled in the art and can also be obtained using, e.g., <http://www.lycos.com>. An overview of patent information in biotechnology and a survey of relevant sources of patent information useful for retrospective searching and for current awareness is given in Berks, TIBTECH 12 (1994), 352-364.

The pharmaceutical compositions, uses, methods of the invention can be used for the treatment of all kinds of diseases hitherto unknown as being related to or dependent on the modulation of tumor suppressor genes or their encoded protein. The pharmaceutical compositions, methods and uses of the present invention may be desirably employed in humans, although animal treatment is also encompassed by the methods and uses described herein.

The Figures show:

Figure 1: Bop1 Sequence and Tissue Distribution

(A) Sequence of Bop1 protein. Cysteine and histidine residues of the seven zinc finger motifs of the C₂H₂ type are boxed. A putative phosphorylation site for cyclin-dependent kinases (Cdks) corresponding to the consensus motif (b/p)(S/T)Pxb located at residues 56-60 is underlined. A putative phosphorylation site for protein kinase A (PKA) at residue 666 is indicated (*).

(B) Schematic representation of Bop1 clones. Clone p2195 and p1270 were derived from the AtT-20 corticotroph tumor cell line. Clone B-16 was isolated from a BALB/c pituitary library and encodes the same protein identified in p2195 and p1270. The coding region of p1270 and B-16 is interrupted at residue 658 by a 630 bp insertion. The sequences at the boundaries of this insertion are displayed in the lower part of the figure and are in excellent agreement with consensus

exon-intron junctions and preserve the reading frame. Restriction sites for EcoR I (R), BamH I (B) and Not I (N) are indicated.

(C) Expression of Bop1 mRNA in mouse tissue. Bop1 distribution was assessed by northern blot analysis of total RNA prepared from different brain regions (olfactory bulb (Olf), frontal cortex (fCx), occipital cortex (oCx), hippocampus (Hip), hypothalamus-thalamus (HyT), brain stem (BSt), cerebellum (Crb) and peripheral tissues (anterior pituitary gland (Pit)), heart (Hea), liver (Liv), stomach (Sto), intestine (Int), kidney (Kid), adrenal gland (Adr), spleen (Spl), lung (Lun)). Ethidium bromide staining of the gel is shown in the insert to document equal and intact amounts of each RNA preparation.

Figure 2: Bop1 and p53 Alter Proliferation of LLC-PK1 and Saos-2 Cells

Anhydrotetracycline(ATc)-regulated expression of Bop1 and p53 was established in LLC-PK1 and Saos-2 cells.

(A) Cell counts of the parent tTA clones (L-tTA and S-tTA) in comparison to Bop1- and p53-expressing LLC-PK1 (L-Bop and L-p53, respectively) and Saos-2 (S-Bop and S-p53, respectively) clones in the presence (+) and absence (-) of ATc.

(B) Bop1 and p53 inhibit DNA-synthesis (BrdU) and cell viability (MTT). For each time point, BrdU incorporation or formazan blue formation were measured in the absence (-) or the presence (+) of ATc.

(C) Growth inhibition by Bop1 and p53 is serum independent. Cells were grown in the presence of the indicated amount of fetal bovine serum (10% or 0.1%) and in the presence (+) or absence (-) of ATc.

(D) Growth inhibition by Bop1 and p53 is reversible. Cells were seeded in Atc-containing medium, grown in the absence of ATc for 2 days before medium was renewed (arrowhead) with medium containing (-/+) or lacking (-/-) ATc.

Figure 3: Bop1 and p53 Inhibit Soft Agar Colony Formation

Bop1 (L-Bop and S-Bop) and p53 (L-p53 and S-p53) clones were grown in the presence of ATc before plating into soft agar at densities of 1×10^5 (No. 1+4), 5×10^4 (No. 2+5) and 2.5×10^4 (No. 3+6) cells per well in six-well plates. The repressor ATc was included in the upper row (+) and was omitted in the lower row (-). For photography on day 10, the soft agar was overlaid with MTT for 4 hr. Pictures shown are representative of three to five independent experiments.

Figure 4: Bop1 and p53 Induce Apoptotic Cell Death

(A) DNA laddering. Genomic DNA was isolated from Bop1 (L-Bop and S-Bop) and p53 (L-p53 and S-p53) expressing clones grown in the presence (+) or absence (-) of ATc for 3 days, centrifugated and soluble DNA was subjected to agarose gel electrophoresis and stained with ethidium bromide.

(B) Fluorescence microscopy of Bop1 and p53 clones stained with ethidium bromide and acridine orange. Cells (a: L-Bop; b: L-p53; c: S-Bop; d: S-p53) were grown in the absence of ATc for 3 days. Floating cells were collected, incubated with ethidium bromide and examined by fluorescence microscopy (510-550nm; x1000).

(C) DNA end labeling. S-Bop (Bop1) and S-p53 (p53) cells were grown for 3 days in the presence (black) or absence (grey) of ATc. Permeabilized cells were subjected to terminal transferase end labeling (TUNEL) in the presence of digoxigenin-labeled dUTP. Cells were then incubated with fluorescein-conjugated antidigoxigenin antiserum and subjected to flow cytometry.

Figure 5: Bop1 and p53 Regulate Cell Cycle Distribution

(A) Induction of G1 arrest by Bop1 and G2/M arrest by p53. S-Bop (upper panels) and S-p53 (lower panels) were grown in the presence (left) or absence (right) of ATc for 3 days. Propidium iodide-stained cells were analyzed by flow cytometry to

determine DNA content. Bop1 reduced the proportion of S-populations in S phase and G2/M phase from 37.8% and 17.5% to 24.5% and 12.6%, respectively and increased cell population in G1 from 44.7% for the repressed state to 63.0% for the expressed state of S-Bop. For p53 a decrease in G1 and S phase from 39.4% to 31.8% and from 43.7% to 35.0% was observed, which was followed by a clear increase in G2/M from 16.9% to 33.2%.

(B) G1-Arrest by Bop1 is independent of p21^{Waf1} expression. S-tTA (tTA), S-p53 (p53) and S-Bop(Bop1) cells were grown in the presence (+) or absence (-) of ATc for 3 days. Western blots of total cell lysates were performed with anti-p21, anti-p53 and anti-GST-Bop1 Δ ZF antisera.

(C) Apoptotic cell death following Bop1 and p53 expression is unrelated to the cell cycle. TUNEL was carried out on permeabilized S-Bop (Bop1, upper panels) and S-p53 (p53, lower panels) cells grown in the presence (left) or absence (right) of ATc for 3 days. Subsequent staining with propidium iodide allowed simultaneous assessment of DNA content and apoptosis by flow cytometry. Grey dots in the boxed area represent cells with high TUNEL fluorescence and hence apoptotic. Dots in different shades of grey outside the boxed area correspond to living cells in G1 (bottom), S and G2/M (top) phase of the cell cycle. Apoptotic fluorescence threshold was set so that less than 5 % of S-tTA cells grown in the presence or absence of ATc were apoptotic. Apoptotic cells in the presence of ATc represent less than 5% of the cells in the case of S-Bop and less than 1% for S-p53. In the absence of ATc, 70% of S-Bop (65% of S-p53 resp.) cells displayed enhanced or high TUNEL fluorescence.

Figure 6: Transfer of PVR1 Gene Regulation through Bop1 Zinc Finger Domain and Nuclear Localization of Bop1

(A) Schematic representation of Bop1/steroid receptor hybrids. Abbreviations used are G and M for human glucocorticoid (GR) and mineralocorticoid receptor (MR) domains, respectively. The transactivation domain of the GR is represented by a hatched box, the MR hormone binding domain by a black box, and the MR

DNA-binding domain by grey box with the two zinc fingers indicated by vertical lines. The numbers above each box indicate amino acids.

(B) The zinc finger domain of Bop1 confers regulation of the PVR1 gene. Native Bop1 and p53 (left) or the hybrid GB_ZM (right) cDNAs were co-transfected with the cAMP-responsive reporter pΔMC16LUC into LLC-PK1 cells (2×10^6) and plated with aldosterone (Aldo; 10^{-9} M) or spironolactone (Spiro; 10^{-7} M). PACAP-38 (10^{-9} M) was added the next day for 4hr before harvesting the cells. To calculate induction ratios, luciferase activity was standardized on MTT values.

(C) Regulation of PVR1 by Bop1 requires transactivation (left). The construct ΔB_ZM is truncated for the GR transactivation domain and was tested under the same conditions as described above. Cytoplasmic trapping of Bop1 prevents transactivation of the PVR1 gene (right). The native Bop1 cDNA was fused to the hormone-binding domain of the MR to create Bop_XM. Transfected LLC-PK1 cells (2×10^6) were replated in charcoal-treated serum and aldosterone or spironolactone were added separately. PACAP-38 (10^{-9} M) was added next day for 4hr before cells were harvested. To calculate induction ratios luciferase activity was standardized with MTT values.

(D) Bop1 is a nuclear protein. S-Bop cells were grown in the presence or absence of ATc for three days and simultaneously immunostained with rhodamine-conjugated phalloidin to stain actin filaments and with a rabbit antiserum raised against a GST-Bop1ΔZF fusion protein. The grey bar represents 25 μm.

Figure 7: Sequences of the ZAC/LOT family

(A) Sequence alignments of the ZAC/LOT proteins. Human ZAC (hZAC, identical to hLOT1), mouse ZAC (mZAC) and rat LOT1 (rLOT) were aligned according to a Clustal method. Residues that matched hZAC with one distance unit are boxed.

(B) Schematic representation of the ZAC/LOT proteins structures. Each domain is indicated as a box: the 7 ZF of C2H2 type, the linker region, the Pro-repeats present in mZAC only, the Pro, Gln and Glu rich region, the Glu-clusters absent in

hZAC, and the C-terminus. The numbering of the aminoacid residues is indicated below the boxes.

(C) Phylogenetic tree for ZAC/LOT family. Only the ZF domains were taken into account for the analysis.

Figure 8: Localizations of hZAC and mZAC genes

(A) Chromosomal localizations. FISH with hZAC probe revealed spots on human chromosome 6 band 6q25; FISH with mZAC probe revealed spots on mouse chromosome 10 band 10A2 and FISH with mZAC probe revealed spots on human chromosome 6 band 6q25.

(B) Southern blots of human and mouse genomic DNA. Human (H) and mouse (M) genomic DNAs were digested with the indicated restriction enzymes, fractionated on a 1% agarose gel and blotted. The blot was first incubated with a radioactive mZAC probe and autoradiographed. Then, the membrane was stripped, the removal of the mZAC probe was checked, and the blot was hybridized with a hZAC probe and autoradiographed. The same data were obtained with another set of enzymes including Nco I, Sac II, Sac I, and Pst I.

Figure 9: Human tissue distribution of hZAC

A human RNA Master blot was hybridized with a hZAC probe and the signal intensity for each dot was measured using a storage phosphor imaging system (Bio-Rad).

There was also a weak expression of hZAC in adult peripheral leucocytes, spinal cord, liver, skeletal muscle and whole brain (less than 150 units). hZAC was expressed in different brain areas: mainly in the occipital lobe, thalamus and cerebral cortex (100-150 units), in other areas (amygdala, caudate, cerebellum, frontal lobe, hippocampus, medulla oblongata, putamen, substantia nigra, temporal lobe, subthalamic nuclei) the signals were very weak (<100 units).

Figure 10: hZAC is a nuclear transcriptional activator

(A) Nuclear localization of hZAC. Immunocytochemistry of SaOs-2 cells transfected with HA-tagged-hZAC (1µg plasmid) or -mZAC (200 ng plasmid) was performed using an anti-HA antibody and a FITC-conjugated secondary antibody. As cells were transiently transfected, positive transfected cells (indicated with an arrow) as well as negative non transfected cells were present on the same sample.

(B) Transcriptional activity of hZAC. SaOs-2 cells were transfected with plasmids encoding a fusion protein between the GAL4 DNA binding domain and either hZAC, mZAC, or the transactivation domains of SP1 or CTF, together with a luciferase reporter gene under the control of a GAL4 sensitive minimal promoter. Luciferase activity for each condition is indicated as a fold stimulation over basal.

Figure 11: hZAC inhibits colony formation

SaOs-2 cells were transfected with the plasmid encoding the puromycine resistance alone (pRK5-PUR), or together with plasmids encoding hZAC, mZAC and p53 in their sense and antisense (as) orientations. Puromycine was added for 9d and the resistant colonies were counted. Mock-transfected SaOs-2 cells were killed by puromycine. This experiment is representative of three independent experiments.

Figure 12: hZAC induces a G1 arrest

(A) Cell cycle distribution. SaOs-2 cells were cotransfected with pRK5 encoding CD20 and different amounts of pRK5 encoding either mZAC, hZAC or p53. CD20 positive and propidium iodide stained cells were analysed by flow cytometry to measure DNA content. This experiment is representative of 3 independent experiments.

(B) Western blots. SaOs-2 cells were transfected with different amounts of pRK5 encoding mZAC, hZAC or p53, as indicated. Western blot of total cell lysates was performed with anti-HA antibody.

Figure 13: hZAC induces apoptotic cell death

DNA laddering: SaOs-2 cells were transfected with different quantities of pRK5 plasmid (1500 ng, lane 1) or encoding CAT (1500 ng, lane 2), mZAC (50, 150 and 500 ng, for lanes 3, 4, 5, respectively), hZAC (500, 1000, 1500 ng, for lanes 6, 7, 8, respectively) or p53 (100ng, lane 9). This experiment is representative of 3 separate ones.

A better understanding of the present invention and of its many advantages will be had from the following examples, given by way of illustration.

Example 1: Cloning, structural analysis and tissue distribution of the TSG Bop 1

In order to isolate DNAs coding for different receptors positively coupled to adenylyl cyclase, we used a recently described expression cloning method (Spengler et al., Nature 365 (1993), 170-175). This method is based on transcriptional induction of a cAMP-responsive luciferase reporter gene by stimulation of adenylyl cyclase through activated target receptors.

Pools of clones from a mouse corticotroph pituitary tumor cell line (AtT-20) (Spengler et al., Nature 365 (1993), 170-175) cDNA library and from a new-born rat colliculi library were co-transfected with a cAMP-responsive reporter into LLC-PK1 cells according to the functional expression transducing cloning technique (*FETCH*).

This expression cloning technique relies on the co-transfection of pools of clones from a cDNA-expression library with a cAMP-responsive reporter into a mammalian cell line, most preferably LLC-PK1 cells.

In a previous series of studies we noted that a cAMP-responsive element derived from the hCRH-gene promoter conferred regulation by cAMP to heterologous promoters (Spengler et al., Mol. Endocrinology 6 (1992), 1931-1941). Further experiments demonstrated, that basal and induced expression depended strictly on the promoter context and the cell line employed. In this view a modified mammary mouse tumor virus promoter (Δ MTV) proved to be exceptional in combining a low level of basal expression with strong induction ratios in various cell lines tested including CV-1 (monkey kidney fibroblast), JAR (human choriochoriocarcinoma), SK-N-MC (human neuroblastoma) and AtT-20 (mouse anterior pituitary) (Spengler et al., Mol. Endocrinology 6 (1992), 1931-1941). Properties of this reporter were further improved by increase of the number of CREs. These modifications allowed a synergistic enhancement in the response to cAMP approaching an induction plateau at a critical number of eight 5' to 3' end inserted copies without change in the basal levels of expression of this construct. Any further extension of the numbers of CREs resulted in an adverse effect due to squelching of TATA-box mediated basal levels of expression. To circumvent this limitation, we constructed by PCR a construct designated p Δ MC16LUC, which contained a duplication of the cAMP-responsive region 5'-CRE₈-TATA-3'.

A panel of cell lines was screened to identify those combining efficient expression from the pRK vector (CMV promoter and CMV enhancer) with high transfection efficiency and with highest responsiveness of the reporter to cAMP. In a preliminary survey, we confirmed in Northern blot experiments that expression from the pRK vector in LLC-PK1 cells was clearly superior to other cell lines used in standard expression cloning techniques e.g. Cos-1 and 293 cells. According to general view highest levels of expression are considered to provide the best chance to detect a specific signal against background noise. Therefore, COS cells are the model of choice in expression cloning strategies allowing replication of transfected cDNAs and resulting in high amounts of proteins of interest, which can be identified by the respective ligand or antibody. Yet, COS cells were poorly responsive to cAMP in regard to induction of the reporter plasmid, so that we investigated in the next step electroporation parameters in LLC-PK1 cells to

obtain high transfection efficiency (number of transfected cells). Electrotransfection parameters (voltage, capacitance, resistance, transfection volume, electrodes, buffer composition) were varied systematically and evaluated semi-quantitatively by *in situ* staining of galactosidase activity of the co-transfected plasmid pCH110, which encodes the β -galactosidase gene under the control of the SV40 promoter. As expected, transient expression levels and transfection efficiency increased linearly to higher field-strengths. In a second series of experiments, we tested the range of induction observed for co-transfection of p Δ MC16LUC with a control plasmid encoding a G-protein coupled receptor expressed from the pRK vector. Importantly, the highest induction ratios obtained deviated clearly from the parameters suggested by *in situ* staining. Strikingly, under conditions revealing expression of the marker protein galactosidase in >80% of the cells the response to cAMP was severely impaired in its amplitude. In contrast, those cells revealing moderate levels of unstimulated luciferase activity with typically 40% of the cells being transfected displayed the strongest induction ratios. This finding was further substantiated by the fact that maximal stimulation of the reporter by endogenous vasopressin receptors of the host cell coincides with those settings derived from transfection of a recombinant cDNA encoding a G-protein coupled receptor. Conclusively, highest sensitivity of this system to cAMP is achieved in case recovery following electroporation is maximized, which will by far outpass any advantage of higher levels of transfection efficiency and higher levels of DNA in individual cells. This correlation is acknowledged in the designation functional expression transductory cloning technique (FETCH) to emphasize that identification of target clones depends on expression of functional (full-length) cDNAs, the presence of which is detected by subsequent activation of an endogenous signal transduction pathway and can be monitored by activation of a downstream amplifier, i.e. the reporter gene.

Additional improvements were introduced to reduce further the extent of cell death during electrotransfection and to permit fastest recovery within the time frame pre-set by the decay of the transfected DNA within 48 hr. At this step, cell density proceeding splitting of the cells and in turn numbers seeded proved to

determine decisively cell viability and viability-independent set-points of cAMP-responsiveness. For instance, transfection of a confluent plate of LLC-PK1 cells resulted in slightly increased cell death but an almost complete loss of cAMP-responsiveness of the reporter due to a dramatic upregulation of basal levels of expression equivalent to the activity obtained under the induced state. This result indicates that cell-cell contact and in-turn mitotic activity of LLC-PK1 cells controls responsiveness of cAMP-dependent transcription factors activated by G-protein coupled receptors. Therefore we developed an empirical scheme to passage LLC-PK1 cells: On day one, cells were seeded at a density of 3.3×10^4 cells/cm² and allowed to grow for 48 hr. Since the doubling time is about 18 hr under exponential growth conditions plates are around 75% confluent on day three, on which medium is renewed. This medium change provides a strong growth stimulus and results 24 hr later in a mild growth arrest due to increasing cell density. Cells for electrotransfection were splitted in the evening at 6.6×10^4 cells/cm² and the release from this growth block allowed an enforced mitotic activity 12 hr later with no visible cell death following electroporation, low levels of basal expression of the reporter and an excellent response to stimulation by cAMP. The cells of the stock population were kept under identical conditions (day 1 seeding 3.3×10^4 /cells cm² cells, day 3 medium renewal, day 4 passaging), which resulted in an accelerated growth behavior. The transition into an optimized transfection competent state required at least two rounds of passages of LLC-PK1 cells under the detailed protocol.

In addition we tested an array of tools described to enhance DNA-uptake and stability (synchronization of cells, butyrate, PEG) or to enhance the responsiveness of the PKA-pathway (Ca^{++} -ionophores, PKC-agonists, phosphatase inhibitors) with all of them influencing adversely sensitivity due to reduced cell viability. A notable exception of this rule was the omittance of serum 8 hr after electrotransfection. Although serum was required immediately after electrotransfection during the recovery phase, one wash and replenishment with serum-free medium in the evening resulted in a 2-3-fold increase in cAMP-

responsiveness of the system, which was attributable to a lowered basal activity of the reporter.

Following transfection of cDNA pools in LLC-PK1 cells stimulation of endogenous vasopressin receptors and activation of the reporter served as an internal control to evaluate responsiveness of the PKA-pathway and in turn cell viability. Though electroporation reveals a higher reproducibility compared to chemical methods slight variations can considerably distort the interpretation of induction ratios because of the above-mentioned dependence on cAMP-responsiveness. In addition we included as a positive internal control a plasmid encoding the β_1 -adrenergic receptor, which is positively coupled to cAMP-production and was expressed from the same expression cloning vector. Identical aliquots of this control plasmid were added to each pool of cDNAs to be tested and a control pool, which was composed of one clearly negative pool of 2,000 independent clones. The combined information of induction ratios for vasopressin and the β_1 -agonist isoproterenol allowed to discriminate between the following situations:

- a) low ratios for vasopressin and isoproterenol point to low cAMP-responsiveness and impaired cell viability.
- b) high ratios for vasopressin and low ratios for isoproterenol point to inefficient transfection or degradation of pool DNA
- c) high ratios for vasopressin and isoproterenol point to optimal transfection.
- d) isoproterenol ratio of test pool below the one of the control pool indicate a number of clones > 2,000 or a poor quality of the DNA.
- e) isoproterenol ratio of test pool above the one of the control pool indicate a number of pools < 2,000 leading to an overestimate in the number of independent clones screened.

In the presented scheme the cut-off for each induction ratio obtained for a substance tested has for each pool to be related to the respective ratios obtained for the external vasopressin and the internal isoproterenol control. In this view a PACAP-dependent induction ratio of 3-fold under condition a) has to be considered significant, whereas under condition c) reflects a borderline value.

This standardization allows to compare different samples from the same or different experimental settings and is a prerequisite to compare results from retesting of borderline pools or from successive subdivisions of a putatively positive pool.

Separate aliquots of cells were incubated with peptide hormones, including PACAP, 12 hr after electroporation. One pool of clones from the rat colliculi library consistently stimulated luciferase activity in the presence of PACAP and a functional clone encoding the PVR1 receptor was isolated by successive subdivisions (Spengler et al., *Nature* 365 (1993), 170-175). Subdivision of the pool of clones was achieved by subdividing the cDNA library until the pool of clones represented a substantially homogeneous pool of clones which consistently stimulated luciferase activity. Several other pools displayed the same phenotype, namely a PACAP-dependent stimulation of the reporter gene and the corresponding active clones were isolated by the same subdivision process. Sequencing was carried out by subcloning restriction fragments in pBSBluescript using T3, T7 and internal primers. Two clones from the AtT-20 library (p2195 and p1270) inducing PVR1 expression turned out to encode the same protein (in the scope of the present invention referred to as Bop1).

The isolated cDNA clones p2195 and p1270 contained a 2.8 kb and 4.7 kb insert, respectively. Entire sequencing of clone p2195 revealed a 2790 bp cDNA (shown in SEQ ID NO. 1) encoding an open reading frame of 667 amino acids (shown in SEQ ID NO. 2) giving rise to a protein with a predicted molecular weight of 75 kDa (Figure 1A). The ATG of AGGCCATGG (SEQ ID NO. 4) was assigned as initiation codon on the basis of its close match to the CC(A/G)CCATGG (SEQ ID NO. 5) Kozak consensus sequence for favored initiation of translation and the presence of an in-frame TGA stop codon 12 nucleotides upstream. Data base searches revealed the presence of seven zinc fingers (Klug and Schwabe, *FASEB J.* (1995), 597-604) in the N-terminal region of Bop1. However, homologies to other members of the zinc finger protein family were low (30% for the best), with the closest group being the GLI-Krüppel family of zinc finger

proteins which have been implicated in normal development and tumor formation (Ruppert et al., *Mol. Cell. Biol.* 8 (1988), 3104-3113). In particular, the first H/C link (HSRERPFKC (SEQ ID NO. 6)) is in good agreement with the consensus motif for the GLI-Krüppel family (H(S/T)GEKP(F/Y)XC (SEQ ID NO. 7)) (Schuh et al., *Cell* 47 (1986), 1025-1032). On the other hand, the remaining 459 C-terminal amino acids displayed no significant homologies to sequences in the Swissprot and NBRF-PIR data bases. The central region of the protein (275-383) is characterized by 34 PLE, PMQ or PML repeats, suggestive of a structure known as poly proline type II helix which is considered to be critically involved in protein-protein interactions (Williamson, *Biochem. J.* 297 (1994), 249-260). The COOH-terminal region is particularly P-, Q- and E-rich, a feature often displayed by transactivation domains of transcription factors. In addition, the presence of a putative phosphorylation site (HSPQK (SEQ ID NO. 8)) for cyclin-dependent kinases (Cdks) located between the second and third zinc finger motif (residues 56-60) as well as a putative PKA-phosphorylation site (KKWTI (SEQ ID NO. 9)) at the very C-terminus (residues 663-666) suggests possible regulation by protein kinases.

Since the cDNAs p2195/p1270 were derived from the AtT-20 tumor cell line there is a potential risk that they harbor mutations which may result in loss or gain of functions not associated with the wild-type form. To rule out this possibility we recloned Bop1 from a plasmid library constructed from whole pituitary tissue of Balb/c mice. To isolate a Bop1 wild-type cDNA, poly(A)⁺ RNA was obtained from 80 male Balb/c mice (Balb/cAnNCrIBR) and reverse transcription was performed on 5 µg poly (A)⁺ with a random primer-NotI adapter (5'-ATGTCTCGAGGCCTTTGCGGCCGCTATANNNNNNNN-3' (SEQ ID NO. 3)). After second-strand synthesis, BstXI adaptors (In-Vitrogen) were added. The cDNAs were digested with Not I, size-selected on a chromaspin column 1000 (Clontech) and cloned into the BstXI /NotI sites of pRK8, a modified pRK5 vector (Spengler et al., *Nature* 365 (1993), 170-175). Screening of ~ 0.5 x10⁶ clones with the p2195 cDNA probe allowed the isolation of one full-length cDNA clone designated B-16, which contained a 3.7 kb insert. Transfection of B-16 into LLC-

PK1 cells successfully substituted for p2195 or p1270 with respect to regulation of PVR1 expression. Entire sequencing of clone B-16 showed a 86 bp non translated 5' region and an extended non translated 3' region of 0.7 kb (Figure 1B). The coding region of B-16 was identical to p2195 except the reading frame was interrupted at residue 658 by a 630 bp insertion. The sequences at the boundaries of this insertion are in excellent agreement with consensus exon-intron junction sequences and preserve the reading frame (Figure 1B). We observed this insertion at exactly the same position in clone p1270 derived from the AtT-20 library (Figure 1B). This finding argues against a cloning artefact in clone B-16 and suggests the presence of an unspliced intron region. In support of this hypothesis, a PCR-based fragment encoding the intron region failed to hybridize to a poly-A⁺ blot from AtT-20 cells. The distribution of Bop1 was assessed by Northern blot of total RNA prepared from different mouse tissues. Interestingly, the anterior pituitary gland displayed by far the highest level of expression of Bop1 mRNA (Fig. 1C). Bop1 gene was expressed at much lower levels in various brain areas including olfactory bulb, cortex, hippocampus, hypothalamus-thalamus, brain stem and cerebellum, while no hybridization was observed in peripheral tissues.

Example 2: Constitutive Expression of Bop1 and p53 Abates Growth of Tumor Cells

In order to study the function of Bop1 we aimed to generate clones stably expressing Bop1 protein in the LLC-PK1 cell line. However, independently of the resistance marker employed, we failed to establish a Bop1-expressing cell clone. To evaluate the possibility that Bop1 inhibits tumor growth we subcloned Bop1 and p53 in sense and anti-sense orientation downstream of a cytomegalovirus promoter in a vector (pCMVPUR) carrying the puromycin resistance gene.

The pCMVPUR sense/antisense constructs (1.0 µg) and pGEM4 filling DNA (3.0 µg) were transfected into 2x10⁶ into the LLC-PK1 cell line and in addition into the

human osteosarcoma cell line Saos-2 (ATCC HTB 85), which was previously shown to be growth-inhibited by wild-type p53 (Diller et al., Mol. Cell. Biol. 10 (1990), 5772-5781). pGEM4 replaced pCMVPUR in mock transfected cells. Three electroporations for each construct were pooled and aliquots were plated in 15 cm culture dishes. The cell lines were grown in DMEM (GIBCO) supplemented with 10% fetal calf serum (GIBCO). Selection with puromycin (5.0 μ g/ml) was started 24 hr after transfection. Following transfection, cells were grown with puromycin for 10 days, and the number of viable colonies was scored after incubation with MTT. Data presented in Table I show that introduction of Bop1 sense expression vectors resulted in a substantial suppression of colony formation equivalent to that induced by p53. Abrogation of cell growth by Bop1 or p53 was more prominent in the Saos-2 cell line. In addition the clones that did appear after transfection of Bop1 or p53 sense constructs into the LLC-PK1 cell line died when reexposed to selection after passaging and grew at a slow rate in case further selection was omitted.

Table I

Bop1 and p53 Suppress the Growth of Tumor Cells

Cell type	(n)	plasmid	antisense	sense	ratio
LLC-PK1	3	Bop1	1014 \pm 170	2	507
	3	p53	1452 \pm 258	2	726
	1	vector	1653 \pm 270		
	1	mock	0		
Saos-2	3	Bop1	2538 \pm 354	1	2500
	3	p53	3779 \pm 566	1	3800
	1	vector	4517 \pm 641		
	1	mock	0		

The epithelial cell line LLC-PK1 and the human osteosarcoma cell line Saos-2 were electrotransfected (n=3) with the parent vector pCMVPUR or with vectors encoding sense and antisense Bop1 or wild-type rat p53. pGEM4 carrier DNA replaced pCMVPUR in mock transfected cells. 24 hr later, cells were grown in the presence of 5 μ g/ml of puromycin and kept for 10 days with regular medium changes. To score viable colonies cells were incubated with MTT.

Example 3: Bop1 and p53 Suppress Growth of Tumor Cells

A system for tetracycline-regulated gene expression was recently described (Gossen and Bujard, Proc. Natl. Acad. Sci. 89 USA (1992), 5547-5551). This system relies on constitutive expression of a tetracycline-controlled transactivator protein (tTA) which activates target genes placed under the control of a regulatory sequence (tetO). Binding of tetracycline (Tc) or its higher affinity derivative anhydrotetracycline (ATc) to tTA prevents activation, whereas activation is achieved by withdrawal of the repressor (Gossen et al., Trends Biotech. 12 (1994), 58-62). In the approach presented here LLC-PK1 and Saos-2 cell lines are transfected with a tTA-encoding vector and isolated one clone from each cell line (L-tTA and S-tTA) which displayed efficient regulation of genes cloned downstream of the tetO sequence.

In addition, a new cis-regulatory expression vector was developed with distinct lower basal levels of expression and potent regulatory properties equivalent to or exceeding those exhibited by the original minimal CMV-based expression vector in a panel of host lines attesting to a broad use of this system in future applications, most preferably the study of TSGs. The regulatory region of pUHC13-3 (Gossen and Bujard, Proc. Natl. Acad. Sci. USA 89 (1992), 5547-5551) was excised by HindIII and EcoRI partial digest and inserted into pBlueScript SK(-) digested by EcoRI and HindIII to give pBS-CMVtetO. A fragment of 157 bp encoding the tetO sequence was released by SmaI and inserted into the plasmid p Δ MTVLUC (Spengler et al., Nature 365 (1993), 170-

175) linearized at +256 bp by HindIII and blunted with T4-DNA polymerase to give the construct p Δ MtetOLUC.

The XhoI site (+1) of p Δ MtetOLUC was converted into a NotI site by insertion of an oligonucleotide with an internal NotI site. To obtain p Δ 5' Δ MtetOLUC a StuI (+863) / NotI (+1) fragment of p Δ MtetOLUC was inserted into pBlueScript cut by SmaI/NotI and shortened in size by PpuMI (+786 bp) and EcoRV digestion, blunting and relegation. This fragment was either transferred back into the plasmid p Δ MTVLUC using the pBlueScript polylinker HindIII site and the internal BstEII site (+56bp) or transferred into the plasmid pOPIPUR by HindIII and NotI digestion to give PMtetO. The vector pOPIPUR is derived from pOPI3CAT (Stratagene) and contains the puromycin gene of pPUR (Clontech) under the control of the SV40 promoter.

Additional copies of the heptameric tetO sequence were isolated from pBS-CMVtetO by SmaI and KpnI digestion and inserted into PMtetOLUC restricted within the tetO sequence by Ecl136II and KpnI. Using this strategy, a series of constructs with increasing numbers of tetO copies was created, which are abbreviated in the following part as PMtetO_xLUC with the index displaying the number of copies of the heptamer tetO.

Target cDNAs were inserted downstream the Δ MtetO sequences via the unique Not I site. For stable transfections the plasmids p3'SStTA, PMtetO₅Bop1 and PMtetO₅p53 were linearized with Eam11051I and 1 μ g of DNA was co-transfected with 3 μ g pGEM4 filling DNA into 2×10^6 cells. Selection of tTA-cell clones started 24 hr after transfection using hygromycin (MERCK) at a concentration of 700 μ g/ml and 500 μ g/ml in LLC-PK1 and SaOs-2 cells, respectively. Selection for clones expressing the Bop1 gene or p53 was carried out at a concentration of 5.0 μ g/ml puromycin. The following numbers of clones were screened: L-tTA: Bop1=95, p53=92 and S-tTA: Bop1 n=77, p53: n=72. All the clones revealed impaired cell growth to varying degrees under the activated state (-ATc), which was microscopically scored twice during seven days. For each condition one half of the most promising clones was subjected to additional rounds of analysis with about 10 clones remaining at the fourth round.

Three candidate clones from each transfection condition were subjected to a preliminary analysis of counts of cell numbers. The LLC-PK1- and Saos-2-derived clones (L-Bop and L-p53, S-Bop and S-p53, resp.) displaying the greatest differences in growth were further analyzed (Figure 2A). Importantly, no major differences in the growth behavior were observed in the presence of the repressor ATc between Bop1- and p53-expressing clones and the parent clones L-tTA and S-tTA (Figure 2A). Therefore the differences in cell counts on day six were primarily due to the suppression of growth in the absence of the repressor. Measurement of proliferation rate revealed that Bop1 (L-Bop: 11-fold; S-Bop: 20-fold) was slightly less potent than p53 (L-p53: 15-fold; S-p53: 25-fold) in reducing the growth rate of both cell lines. Western blot analysis proved that Bop1 protein was not detectable in L-Bop or S-Bop cells in the presence of ATc. A strong increase in protein levels of Bop1 was noted in the activated state (Figure 5B). Similar results were also obtained for the regulation of p53 in Saos-2 and LLC-PK1 cells (Figure 5B). These results emphasize that the modified expression vector combines low basal activity with potent regulatory properties.

Total counts of cell numbers do not necessarily discriminate between alteration of cell proliferation and viability. It was therefore decided to evaluate the effects of Bop1 and p53 expression by two complementary methods. First, DNA-synthesis was studied with a non-radioactive immunoassay based on incorporation of 2-bromodeoxyuridine (BrdU) into nuclear DNA on each of six days with or without ATc (Figure 2B). Second, it was measured the conversion of the tetrazolium salt MTT to formazan blue, which depends on the activity of mitochondrial and cytoplasmatic dehydrogenases. This activity depends on cell viability and closely correlates with cell proliferation (Figure 2B).

The counts of Cell Numbers, 2-Bromodeoxyuridin incorporation and Formazan production were performed as follows:

Equal number of cells (5,000) were seeded in 24-well plates in DMEM / 10%FCS supplemented with ATc (10^{-11} μ g/ml). After recovery for 36 hr, medium was renewed and the repressor omitted for half of the samples. For samples lacking the repressor, the medium was changed again 3 hr later to remove residual

amounts of ATc. Growth medium was changed routinely on day 3. Average cell counts from 3 to 5 experiments in triplicate are plotted versus time after removal of the repressor. For measurement of DNA-synthesis cells (1,000) were seeded in 48-well plates and cultured as outlined above. On each of six days, 10 μ M 2-bromodeoxyuridin was added for 8 hr and subsequent steps were carried out according to the manufacturer's instructions (Boehringer Mannheim). For measurement of cell viability, 1,000 cells were seeded in 24-well plates and cultured as described above. The average of OD measurements for DNA-synthesis and cell-viability was obtained from three experiments performed in triplicates. To test serum-independence, cells were kept in normal medium for 36 hr before serum was washed out once with DMEM and replaced by DMEM / 0.1%FCS / \pm ATc.

The results obtained for S-Bop and S-p53 emphasize the observed differences in cell counts (Figure 2A), which correlate with those obtained in overall cell proliferation and overall viability measurements (Figure 2B). Similar results were obtained for L-Bop and L-p53. Cells from LLC-PK1 and Saos-2 clones kept under low serum conditions (0.1%FCS) in the repressed state displayed reduced growth rate and cell death from day three on, indicating serum-dependence to maintain logarithmic growth (Figure 2C). In contrast, proliferation under expression of Bop1 and p53 remained unchanged (Figure 2C). Therefore, inhibition of tumor growth by Bop1 and p53 proceeds through mechanisms unrelated to the presence of serum factors in these cellular models.

The ability of Bop1 to suppress growth could be due to a non-specific lethal effect of protein overproduction, resulting in cell death. Alternatively, it could be a manifestation of a more specific effect on cell proliferation. To further investigate these two possibilities, the growth pattern following reexposure to ATc of the surviving cells was tested. The impairment of cell growth by Bop1 and p53 expression was transient for both the LLC-PK1 and Saos-2 clones studied. Reexposure to the repressor ATc caused cells to resume logarithmic growth after 48 hr (Figure 2D). Therefore, Bop1- and p53-induced changes in cell growth were

not permanent and at least in part reversible, arguing against a non-specific effect of protein overproduction.

Example 4: Bop1 and p53 Inhibit Soft-Agar Colony Formation

Anchorage-independent growth is often correlated with tumorigenesis and is a strong criteria for cultured cell transformation. To test the influence of Bop1 or p53 on anchorage-independent growth, LLC-PK1 and Saos-2 cell clones were assayed for their ability to grow in soft-agar. Each well (35-mm) of a six-well culture dish was coated with 4 ml of bottom agar mixture (DMEM/10%FCS/0.6% agar/ \pm ATc). After the bottom layer had solidified, 2 ml of top agar mixture (DMEM/10%FCS/0.3%agar/ \pm ATc) containing the cells was added. ATc was used at a final concentration of 3×10^{-11} μ g/ml. After 7 days, another 1.5 ml top agar mixture (\pm ATc) was added. On day 10, the wells were overlaid with 2 ml MTT (1mg/ml) and incubated for an additional 4 hr, washed once with PBS and then photographed. Colony formation by Bop1 or p53 expressing cells (-) was dramatically reduced compared to the repressed state (+) (Figure 3). Also the few colonies formed under Bop1 or p53 expression were of smaller size. These results demonstrate that Bop1 and p53 can abate anchorage-independent growth of tumor cells, one of the hallmarks of tumorigenicity.

Example 5: Bop1 and p53 Suppress Tumor Formation in Nude Mice

The most stringent experimental test of neoplastic behavior is the ability of injected cells to form tumors in nude mice. Yet, not all of the altered cellular growth properties commonly associated with the transformed state in-vitro are required for neoplastic growth in-vivo and vice versa. Therefore loss of tumorigenicity under expression of Bop1 in-vivo would be a critical test to substantiate the tumor suppressor function of Bop1. To achieve gene regulation by Tc in nude mice, half of the animals were implanted with Tc pellets whereas the remainder were implanted with placebo pellets. 36 nude mice were randomly

distributed into three groups of 12 animals. In each group, half of the animals were subcutaneously implanted with Tc pellets (63 mg; 0.7 mg tetracycline hydrochloride per day; Innovative Research of America) and the remaining half were implanted with the placebo pellets (Innovative Research of America). Two days latter, each animal was injected subcutaneously on each side with S-Bop or S-p53 cells which were grown in the presence of ATc, trypsinized and resuspended in PBS at a density of 5×10^7 cells/ml. 100 μ l of this cell suspension was injected subcutaneously into each side of each animal grown in the continuous presence of ATc. Two groups were injected with S-Bop cells from two independent trypsinizations whereas one experiment was performed with S-p53 cells. Due to the clonal origin of S-Bop and S-p53, differences in the tumorigenicity of each clone were observed as inferred from the difference in the observed lag in tumor formation which was assessed at 11 weeks after cell injection for S-Bop and at 16 weeks for S-p53. S-Bop- and S-p53-injected animals were sacrificed at 11 and 16 weeks, respectively, dissected and the tumors were weighed. Table II presents results from two experiments with S-Bop (Bop1) and one experiment with S-p53 (p53). In agreement with previous results (Chen et al., Science 250 (1990) 1576-1580), p53 expression impaired tumor formation by Saos-2 cells in-vivo. Interestingly, Bop1 was as efficient as p53 in inhibiting tumor formation as deduced from tumor incidence (Table II) and from the average tumor weight (193 ± 13 mg ($n=14$) for Tc vs. 18 ± 7 mg ($n=2$) for placebo). Conclusively, Bop1 and p53 are equipotent at inhibiting tumor formation in-vivo.

Table II

Bop1 and p53 Inhibit Tumor Formation in-vivo

clone	tumor incidence (No. of tumor-bearing injection sites / total No. of injection sites)	
	placebo	Tc
S-Bop (Bop1) exp. n°1	2/12	14/14
S-Bop (Bop1) exp. n°2	1/12	12/12
S-p53 (p53)	1/12	10/12

Nude mice were implanted with placebo or Tc pellets subcutaneously. Two days latter, 5×10^6 cells from each clone were injected subcutaneously into each side of each animal, and tumor formation was scored at 11 weeks for S-Bop (Bop1) and 16 weeks (p53).

Example 6: Expression of Bop1 and p53 induce apoptosis

Two days following induction of p53 expression, Saos-2 cells flattened and greatly enlarged (three to eight fold) in average diameter, which was most evident when grown in small clusters. Similar changes, though less prominent (two to fourfold increases in the average diameter), were also observed for L-p53. In contrast, Bop1 expressing LLC-PK1 or Saos-2 clones appeared indistinguishable from the parent cell lines giving a first hint of functional differences between Bop1 and p53. Yet, an increasing number of cells with signs of lost cell viability was observed from day two onwards following Bop1 or p53 expression. These cells failed to convert MTT, shrank in size, were abundant in phase contrast microscopy, revealed membrane blebbing, and rounded further up before detaching from the plates. For Bop1 these alterations were most evident in Saos-2 cells (S-Bop) and

for p53 in LLC-PK1 cells (L-p53) and appear reminiscent of an apoptotic cell death. This form of cell death is often accompanied by fragmentation of the DNA into a ladder of regular subunits.

To address this question LLC-PK1 and Saos-2 cells were seeded with (4,000 cells/cm²) or without (8000 cells/cm²) ATc for 3 days and soluble DNA was prepared as described (Hockenbery et al., Nature 348 (1990), 334-336). Aliquots of DNA were fractionated on a 1.2 % agarose gel. When the repressor was omitted a clearly visible degradation into oligonucleosomal DNA fragments became evident (Figure 4 A), which was most advanced following expression of Bop1 in Saos-2 cells.

The fluorescent DNA-stains ethidium bromide and acridine orange were employed to examine nuclear changes under the ATc-deprived condition. Therefore, the cells (5x10⁴) were seeded in the absence of ATc in 12-well clusters and grown for three days. After aspirating the medium, the cells were washed twice with PBS and overlaid with a staining mix of ethidium bromide (50 µg/ml) and acridine orange (10 mg/ml) for 10 - 20 min. Photography was carried out using UV-filters of 400-420 nm and of 510-550 nm.

Since the flattened and enlarged cell shape of p53-expressing cells enhanced attachment to the plastic surface, a comparable large population of cells exhibited nuclear signs of apoptosis, whereas Bop1-expressing cells shrank, dislodged quickly and appeared less represented in these experiments. The structural changes of nuclear demise following Bop1 expression were even more evident when floating cells were collected and subjected to analysis (Figure 4B). Decay of the nucleus involved nuclear shrinkage, condensation of the chromatin, collapse into patches and then into crescents in tight apposition to the nuclear envelope, and finally in one or several dense spheres (Figure 4B).

To investigate the extent of DNA-damage, terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) was performed using the ApopTag kit (Oncor), according to the manufacturer's instructions, followed by flow cytometry. The cells were seeded with (1,800 cells/cm²) or without (3,600 cells/cm²) ATc for 3 days. Then the cells were pelleted, kept on ice for at least 10 min and

resuspended in 900 μ l of propidium iodide staining solution (PISS = 50 μ g/ml propidium iodide; 0.1 % trisodium citrate dihydrate; 0.1 mg/ml RNase A; 0.1 % Triton X-100). Following an overnight incubation, cell cycle phase distribution was determined with FACScan (Becton-Dickinson) with 20,000 events analyzed using Modfit software (Verity Software House, Inc.). Incubation of each clone in the absence of ATc induced a large increase in the number of cells with enhanced or high fluorescence indicative of free DNA ends and nuclear-fragmentation (Fig. 4C). These results confirm the data obtained with ethidium bromide-stained gels and indicate that the proportion of cells displaying nuclear damage was as high as 60-70 % following expression of either Bop1 or p53.

Taken together these experiments give convincing evidence that Bop1 and p53 recruit apoptotic programs to inhibit growth of tumor cells and Saos-2 cells seem highly apoptosis proficient following expression of Bop1.

Example 7: Expression of Bop1 and p53 induces changes in cell cycle distribution

To characterize further the mechanisms by which Bop1 might regulate cell growth the distribution of cell cycle phases was studied. Increases in wt p53 levels are known to suppress cell growth by blocking the cell cycle at the G1 to S transition (Hunter and Pines, Cell 79 (1994), 573-582; Sherr and Roberts, Genes and Dev. 91 (1995), 1149-1163). More recently p53 has been suggested to address an additional checkpoint by arresting cells at the G2/M boundary (Agarwal et al., Proc. Natl. Acad. Sci. USA 92 (1995), 8493-8497; Cross et al., Science 267 (1995), 1353-1356; Stewart et al., Oncogene 10 (1995), 109-115; Yamato et al., Oncogene 11 (1995), 1-6). In control experiments, the parent clones L-tTA and S-tTA showed no difference in the distribution of cells in different phases of the cycle in the absence or presence of ATc. In contrast, expression of Bop1 reduced the proportion of S-Bop populations in S phase and G2/M phase from 37.8 % and 17.5% to 24.5 % and 12.6 %, respectively. Importantly there was a clear

compensatory increase of cell populations in G1 from 44.7 % for the repressed state to 63.0 % for the expressed state of S-Bop (Figure 5A).

The results obtained for p53 expression in the S-p53 cell clone are in agreement with those obtained recently with a temperature-sensitive mutant p53 in Saos-2 cells (Yamato et al., *Oncogene* 11 (1995), 1-6). A decrease in G1 and S phase from 39.4 % to 31.8 % and from 43.7% to 35.0 % was observed and a clear increase in G2/M from 16.9 % to 33.2 %. (Figure 5A). The failure of p53 to proceed to a G1 arrest reflects most likely the presence of the deleted non-functional retinoblastoma gene product (Rb) in the Saos-2 cell line.

These observations were extended to the LLC-PK1 cell line and though shifts of populations in cell cycle phases under expression of Bop1 and p53 were less prominent than in the Saos-2 cell clones, there was again a clear increase in G1 phase populations for expression of Bop1 (G1 59.1 % vs. 43.7 %; S 28.2 % vs. 38.9 %; G2/M 12.7 % vs. 17.4 %) and a shift for G2/M populations under p53 (G1: 39.3 % vs. 44.1 %; S: 32.1 % vs. 40.2 %; G2/M: 28.6 % vs. 15.7 %).

p53 achieves G1 arrest through transactivation of the gene encoding the cyclin-dependent kinase inhibitor p21 (also designated Cip1, Waf1, Sdi1, Cap20). Increased levels of p21 inhibit the kinase activity of cdk2 and maintain Rb in its underphosphorylated state in tight association with members of the E2F family. As a result, transactivation of genes driving the cell cycle is inhibited (Goodrich et al., *Cell* 67 (1991), 293-302; Weinberg, *Cell* 81 (1995), 323-330). The question arose whether Bop1-induced G1 arrest utilizes the same molecular pathway as p53. Expression of p53 in Saos-2 cells resulted in a strong induction of the p21 protein proving an intact and efficient transactivation of the endogenous gene by the exogenous p53 protein (Figure 5B). Yet, no regulation of the p21 gene in Saos-2 cells was encountered following expression of Bop1 (Figure 5B). The same results were obtained in the LLC-PK1-clones with a strong induction of p21 by p53. Conclusively, Bop1 induces G1 arrest in these cellular models through molecular relays independent of p21.

In a number of cellular systems, wt p53 activation has been shown to confer growth arrest (Mercer et. al., *Proc. Natl. Acad. Sci. USA* 87 (1990), 6166-6170;

Merlo et al., *Oncogene* 9 (1994), 443-453; Michalovitz et al., *Cell* 62 (1990), 671-680; Roemer and Friedmann, *Proc. Natl. Acad. Sci. USA* 90 (1993), 9252-9256). In contrast, wt p53 failed to cause a measurable arrest in M1 cells and cell cycle progression proceeded while viability was lost within 48 hr (Yonish-Rouach et al., *Mol. Cell. Biol.* 13 (1993) 1415-1423; Yonish-Rouach et al., *Nature* 352 (1993) 345-347). In that system, cells in G1 appeared to be preferentially susceptible to the death-inducing activity of wt p53. Therefore the question arose whether in the used cellular models, in which Bop1 and p53 play a dual role in regulation of apoptotic cell death and cell cycle progression, a particular phase of the cycle is associated with protection or increased susceptibility to cell death. To address this issue the cell cycle analysis was extended and double staining with propidium iodide was performed to measure DNA content and TUNEL to assess apoptosis. As shown in Fig. 5C, apoptotic cells proceeded from each phase of the cell cycle as indicated by the distribution of DNA content of apoptotic cells. It was concluded that cell cycle arrest is not a prerequisite to apoptosis and that both Bop1 and p53 induced apoptosis through a pathway which is independent of the one involved in cell cycle arrest.

Example 8: Bop1 is a nuclear transcription factor

Structural analysis of Bop1 demonstrated features compatible with a transcription factor composed of a N-terminal seven zinc finger DNA-binding domain and a COOH-terminal transactivation domain. Without information on the actual cis-regulatory sequences recognized by Bop1 to transactivate target genes, it was decided to use the induction of the endogenous PVR1 gene as a model to dissect functional domains of Bop1. A bimodal regulation of the PVR1 gene was observed, indistinguishable for Bop1 and wt p53 cDNAs as measured by induction of the cAMP-sensitive luciferase gene (Figure 6B). The decrease in PVR1 expression with high amounts of cDNAs was unrelated to cellular toxicity. The two-zinc finger domain of the hybrid steroid receptor GM₂M (Rupprecht et al.,

Mol. Endocrinology 7 (1993), 597-603) was replaced with the seven-zinc finger domain of Bop1 (B_z) to create GB_zM (Figure 6A).

The hormone-binding domain of the mineralocorticoid receptor was replaced in this construct to avoid possible pleiotropic effects associated with glucocorticoids.

The GR_{NX} , MR_{NX} and GM_zM constructs were previously described (Rupprecht et al., Mol. Endocrinology 7 (1993), 597-603). Primers used to create GB_zM were:

5'-gtgatgcccgcgCCATTCCGCTGTCAAAAATGTG-3' (+7 bp to +27 bp) (SEQ ID NO. 10)

and 5'-ccgcgcctcgagGGTCTTCTTGGTGTGACG-3' (+618 bp to +601 bp) (SEQ ID NO. 11).

The different constructs were subcloned into pRK5PUR. To create the construct ΔB_zM , the GR-transactivation domain and part of the Bop1 zinc finger binding domain was excised from GB_zM by EcoRI/MluI digestion and replaced by the restriction fragment EcoRI/MluI (-541 bp to +272 bp) of p2195.

Primers used to create Bop_zM were:

5'-gcggccgCAGAGCCGTCTTTCACTC-3' (+1148 bp to +1166 bp) (SEQ ID NO. 12) and

5'-ccgcgcctcgagAACTGTCCATTTCTTATAGAC-3' (+2001 bp to +1980 bp) (SEQ ID NO. 13).

The stop codon of p2195 was replaced by the amino acid histidine (CTC) as part of the XhoI site used to ligate to the MR-hormone binding domain. PCR-generated fragments were sequenced to verify accurate amplification.

In transfection of LLC-PK1 cells (2×10^6), pGEM4 plasmid was used as carrier and the amount of pRK expression vector was kept constant with pRK5CAT. Luciferase activity was determined as previously described (Spengler et al., Nature 365 (1993), 170-175) 12 hours after transfection.

The Bop1/steroid-receptor hybrid gene GB_zM was co-transfected with the cAMP-responsive reporter p $\Delta MC16LUC$ into LLC-PK1 cells. Aliquots of transfected cells were incubated either with the mineralocorticoid receptor antagonist spironolactone or the agonist aldosterone and PACAP was added to both

conditions after 12 hr (Figure 6B). Though the transactivation potency of GB₂M was 10-fold less compared to the native Bop1 cDNA, a bimodal induction of the PVR1 gene for increasing amounts of GB₂M was consistently observed, which closely paralleled the one observed for Bop1 and p53. In contrast the construct Δ B₂M, which lacks the glucocorticoid receptor transactivation domain failed to confer regulation of PVR1, implicating an active transcriptional mechanism underlying this response (Figure 6C). No regulation of the PVR1 gene was observed for the transfected parent construct GM₂M.

Further support for the role of Bop1 as a nuclear transcription factor was obtained with the fusion protein Bop_xM, in which the C-terminus of Bop1 was linked with the hormone-binding domain of the mineralocorticoid receptor (Figure 6A). Transfection of this construct into LLC-PK1 cells completely prevented transactivation of the PVR1 gene in the absence of mineralocorticoid receptor ligands. In contrast aldosterone and spironolactone allowed efficient regulation of the PVR1 gene (Figure 6C). The activation of Bop_xM by the aldosterone antagonist spironolactone supports the view that the attached hormone-binding domain merely serves to trap this fusion protein to cytoplasmic heat shock proteins (Picard, Trends Cell Biol. 3 (1993), 278-280) and does not interfere otherwise with the functions of Bop1. In contrast, release of Bop1 from this cytoplasmic anchor by either aldosterone or spironolactone allowed nuclear translocation and transactivation of Bop1 targeted genes.

Moreover, to prove nuclear localization of Bop1 immunocytochemistry on S-Bop cells was performed with an antiserum that was raised against a Bop1 fusion protein truncated for the zinc finger domain (GST-Bop Δ ZF).

The plasmid encoding the GST-Bop Δ ZF fusion protein was constructed by a partially digesting the plasmid pRK8-p2195 with BstX I, blunt-ending with T4 DNA polymerase and digesting with Not I. The resulting 0.9kb fragment was subcloned into pGEX-5X-3 (Pharmacia) previously digested with Sma I and Not I. The fusion protein was purified by affinity chromatography using glutathione-sepharose beads followed by SDS-PAGE and electroelution. Rabbits were immunized with

40 µg of the fusion protein and antisera were collected on a weekly basis. Purified IgG were used for western blots and immunocytochemistry experiments. Western blots were performed on total cell lysates (50 µg) with the above-mentioned purified IgG or with commercially available antibodies to p53 (Pharmingen, San Diego, USA catalog # 14091A), p21^{Waf1} (Transduction laboratories, Lexington, USA, catalog # C24420), p27^{Kip1} (Transduction laboratories, catalog # K25020) and p16^{ink4} (Santa Cruz Biotechnology, Inc., Santa Cruz, USA, catalog # sc-759). Immunocytochemistry, and labeling and staining of actin filaments with rhodamine-conjugated phalloidin were performed as previously described (Ibarrondo et al., Proc. Natl. Acad. Sci. USA 92 (1995), 8413-8417). As shown in Figure 6D, no Bop1 immunoreactivity was detected in the presence of ATc whereas an intense nuclear immunostaining was seen in the absence of Atc.

A nucleic acid molecule prepared by the process described herein is exemplified by a culture deposited in the culture collection Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH in Braunschweig, Germany on August 12, 1996, and identified as:

pBluescript II SK (-) p2195 (NotI).

This culture was assigned accession number DSM 11112.

Example 9: Cloning of the human homolog of Bop1

As has been shown in the examples above, Bop1 (in the following referred to as mZac for mouse zinc finger protein which shared with p53 the ability to regulate concomitantly apoptosis and cell cycle progression) is the first gene beside p53 which regulates the two fundamental genetic programs, i.e. cell cycle progression and apoptosis; see Examples 6 and 7. The inventors therefore expected that mZAC should also share with p53 its tumor suppressor activity and isolated the human homolog of mZAC in order to investigate whether human tumors could harbor inactivating mutations of ZAC. Furthermore, hZAC could display otherwise

functional inactivation by methylation of both alleles or imprinting of one allele and loss of the remaining alleles as has been shown for the cdk inhibitor p16 and the new TSG p73 (Merlo et al., *Nature Medicine* 1(7) (1995), 686-692; Kaglrad et al., *Cell* 90(4) (1997), 809-819). Therefore, studies on ZAC expression in human tumors are under progress. In addition, functional inactivation of the ZAC gene in human tumors could rely on genetic mechanisms described so far observed only in genetic diseases, such as minisatellite instability in coding and noncoding parts of the ZAC gene (Buard and Jeffrey, *Nature Genetics* 15 (1997), 327-328). The human counterpart of mZAC (hZAC) was isolated from human brain and pituitary cDNA libraries. 1×10^6 clones from a human pituitary gland cDNA library (5'-STRETCH, Clontech) were screened with a random-primed probe corresponding to the coding sequence of SEQ ID NO: 1 using standard procedures. 40 positive clones were subcloned into pBlueScript and sequenced on both strands using T3, T7 and internal primers. One 2.3 kb clone comprising the nucleotide sequence given in SEQ ID NO: 16 contained the full coding sequence of hZAC (1389 bp, encoding the amino acid sequence as depicted in SEQ ID NO: 17), with a 802-bp 5' untranslated region and a 142-bp 3' untranslated region. Sequence alignments and phylogenetic trees were performed using Lasergene software, DNASTAR Inc., Madison, WI.

The overall identity between hZAC and mZAC coding sequences was 74.6% at the nucleotide level and 68.5% at the amino acid level. Several domains could be identified in hZAC, each having its counterpart in mZAC (Fig. 7A, 7B). The 7 (C2H2) type zinc fingers (ZF) domain at the N terminus was the most conserved (84.2% similarity at the amino acid level); it displayed the same consensus phosphorylation site for Cyclin-dependent kinases (HSPQK, SEQ ID NO: 8) between the second and third ZF (residues 56-60). The linker region, the Pro, Gln and Glu rich region and the C terminus (Fig. 7B) were rather conserved (54%, 58% and 63% identity, respectively). The last 11 C terminal residues were identical (Fig. 7A). There were two major differences between the mouse and human sequences. Indeed, two regions of mZAC were missing in hZAC: a 34 Pro-repeats (PLE, PMQ or PML) domain and a Glu-clusters domain (Fig. 7A, 7B).

A FASTA analysis for homology search in the Uni-Gene database (05/29/97, (Pearson, Proc. Natl. Acad. Sci. USA 85 (1988), 2444-2448) indicated that hZAC sequence matched 43 human expressed sequence tags derived from various adult sources including placenta, aorta, ovary, prostate, heart, as well as fetal tissues including heart, brain, cochlea, liver and spleen. During the course of this study, a rat and a human sequences designated LOT1 (accession no. U72620 and U72621) were reported (Abdollahi, Oncogene 14 (1997), 1973-1979, Abdollahi, Cancer Res. 57 (1997), 2029-2034) and displayed significant homology to hZAC. Indeed, hZAC and hLOT1 were identical except at 2 residues (Leu⁸¹ in hZAC and rLOT1 is a Phe in hLOT1 and Pro⁴⁴⁰ in hZAC (Pro⁶⁷¹ in rLOT1) is an Ala in hLOT1). Surprisingly, the 5'-untranslated regions of hLOT1 and hZAC were completely different 189 nucleotides upstream the ATG. One explanation for this discrepancy is that hZAC or hLOT1 5' ends could correspond to two different splice variants or either one could contain an unspliced intron. The second hypothesis was favored as a putative 3' splicing site (CACAG) was present 190 nucleotides 5' of the ATG in hLOT1. Furthermore, an intron was also present at that position in the mouse gene.

Since large domains of mZAC were missing in hZAC, the existence of additional genes closely related to mZAC was evaluated, which could contain these domains. Extensive PCR analysis of human genomic DNA was performed with degenerate primers, corresponding to residues conserved between hZAC and mZAC in the ZF domain and the Pro and Gln rich region. Only two classes of PCR fragments different from ZAC were isolated but suggestive of closely related genes. During the course of this study, sequences of the corresponding cDNAs became available in the Unigene database. One class of PCR fragments corresponded to hPLAG1, an embryonic gene which could be involved in the pathogenesis of pleiomorphic adenomas of the salivary glands (Kas, Nature Genet. 15 (1997), 170-174). The second class of PCR fragments corresponded to KIAA0198, a cDNA cloned from human cell line KG-1, whose function is still unknown (Nagase, DNA Res. 3 (1996), 17-24). Multiple sequence alignment

revealed that these cDNAs are members of the same new family of ZF proteins (Fig. 7C).

Example 10: Chromosomal localizations and Southern blots of genomic DNAs

First chromosomal localization of both genes were performed by fluorescent in situ hybridization (FISH). The 2.3 kb cDNA of hZAC, the 4.7 kb cDNA of mZAC and the 6 kb BglII fragment of mZAC gene were used as probes, labeled by nick translation with biotin-11-dUTP and hybridized to human and mouse chromosomes as previously described (Eychène, *Oncogene* 7 (1992), 1657-1660). Detection of hybridization was performed using goat anti-biotin antibodies (Vector laboratories, Burlingame, CA) and rabbit FITC conjugated anti-goat antibodies (Biosys, Compiègne, France). Direct banding of BrdU substituted chromosomes (Lemieux, *Cytogenet. Cell Genet.* 59 (1992), 311-312) was stained with propidium iodide for human chromosomes and DAPI for mouse chromosomes. Metaphases were observed under a fluorescent microscope (DMRB, Leica, Germany). Images were captured using a cooled photometrics CCD camera and Quips-smart capture software (Vysis).

The hZAC cDNA probe revealed recurrent single and double spots on human chromosome 6 (band 6q25); out of 30 metaphases, 70% exhibited at least one spot in this position (Fig. 8A). cDNA and genomic mZAC probes exhibited recurrent spots on mouse chromosome 10 (band 10A2); out of 25 metaphases, 50% showed recurrent simple and double spots in this position with the cDNA probe and 60% with the genomic probe, with low background (Fig. 8A). The mouse genomic probe revealed recurrent single spots on human chromosome 6 band 6q25 with the rate of 20% with low background (Fig. 8A). Since mouse 10A2 and human 6q25 loci are syntenic (Copeland, *Science* 262 (1993), 57-66) and since the mouse probe displayed the same spots as the human probe on human 6q25, mZAC and hZAC were either orthologs or related genes clustered in syntenic regions. This data was confirmed by the FISH mapping of hLOT1 which

was recently published (Abdollahi, *Oncogene* 14 (1997), 1973-1979). According to the human genome map (Dib, *Nature* 380 (1996), 152-154, Schuler, *Science* 274 (1996), 540-546), hLOT1 (hZAC) was located between markers D6S308 and D6S978 at 6q24. Chromosome 6 is the fourth most frequently rearranged chromosome in human tumors (Teyssier, *Anticancer Res.* 12 (1992), 997-1004). Allelic loss at 6q24 has been reported in B-cell non-hodgkin's lymphomas (Zhang, *Genes Chromosomes Cancer* 18 (1997), 310-313, Johansson, *Blood* 86 (1995), 3905-3914) and many solid tumors such as gastric carcinomas (Queimado, *Genes Chromosomes Cancer* 14 (1995), 28-34), pancreatic adenocarcinomas (Griffin, *Cancer Res.* 55 (1995), 2394-2399), renal cell carcinomas (Thrash-Bingham, *Proc. Natl. Acad. Sci. USA* 92 (1995), 2854-2858), astrocytomas (Liang, *Neurology* 44 (1994), 533-536), melanomas (Millikin, *Cancer Res.* 51 (1991), 5449-5453, Walker, *Int. J. Cancer* 58 (1994), 203-206), ovarian carcinomas (Lastowska, *Cancer Genet. Cytogenet.* 77 (1994), 99-105, Foulkes, *Br. J. Cancer* 67 (1993), 551-559) and breast cancers (Fujii, *Genes, Chromosomes & Cancer* 16 (1996), 35-39, Noviello, *Clinical Cancer Res.* 2 (1996), 1601-1606, Theile, *Oncogene* 13 (1996), 677-685). Moreover, a frequently deleted region at 6q24 defined by D6S292-D6S310-D6S311 around hZAC locus has been identified in breast carcinomas (Noviello, *Clinical Cancer Res.* 2 (1996), 1601-1606). In addition, microcell-mediated transfer of a defined chromosome 6q fragment around D6S310, thus including hZAC locus, has been shown to suppress the tumorigenicity of the breast cancer cell line CAL51 (Theile, *Oncogene* 13 (1996), 677-685). These genetic data, suggesting that at least one TSG is localized close to hZAC locus, together with the functional data of the present invention point to hZAC as a candidate TSG involved in breast cancer and located at 6q24.

The data obtained in accordance with the present invention have been strengthened by a study recently published by Abdollahi (Abdollahi, *Cancer Res.* 57 (1997), 2029-2034) who cloned ZAC through its loss of expression in a rat model of epithelial ovary cancer and thus named it "LOT" for "Lost On Transformation" (Abdollahi, *Cancer Res.* 57 (1997), 2029-2034). They further isolated hLOT1, which was identical to hZAC, and showed that hLOT1 (hZAC)

expression was also lost in some human ovary cancer cell lines (Abdollahi, Oncogene 14 (1997), 1973-1979). This again suggests that hLOT1 (hZAC) is a candidate TSG.

To further confirm mZAC and hZAC were orthologs, we carried out Southern blots of digested human and mouse genomic DNAs. Human genomic DNA from peripheral blood lymphocytes (obtained from the Montpellier blood bank, France) and mouse genomic DNA from liver were prepared according to standard protocols. Southern blots of digested genomic DNAs were first hybridized with a mZAC probe corresponding to mZAC ZF and linker regions. After autoradiography, blots were stripped and reprobed with a hZAC probe corresponding to hZAC ZF and linker regions. Probes derived from the ZF domains of mZAC and hZAC hybridized to exactly the same bands in both human and mouse DNA (Fig. 8B), indicating that both mZAC and hZAC probes recognized only one gene in both species.

Example 11: hZAC expression in human tissues

hZAC mRNA distribution was determined using a Human RNA Master Blot (PT3004-1, Clontech, Palo Alto, CA) containing normalized amounts of polyA⁺ RNAs from human adult and fetal tissues hybridized with a hZAC probe according to the manufacturer instructions. Since polyA⁺ RNA samples (80 to 400ng) on Master blot have been normalized to the mRNA expression levels of eight different house keeping genes, the relative expression levels of hZAC mRNA could be assessed. hZAC was widely expressed in both adult and fetal tissues (Fig. 9). The strongest levels of expression were observed in the pituitary gland, kidney, placenta, adrenal gland. Uterus, mammary gland, ovary, lung, gastrointestinal tract and lymphoid tissues also revealed strong hybridization signals. Skeletal muscle, peripheral leucocytes, liver, whole brain and spinal cord weakly expressed hZAC. In adult brain, the strongest signals were observed in the occipal lobe, the cerebral cortex and the thalamus (see legend of Fig. 9).

Example 12: hZAC is a nuclear transactivator

The human osteosarcoma cell line SaOs-2 was grown and electroporated as described in Example 2. Except for the GAL4 fusion proteins, all cDNAs were subcloned into the pRK5 vector (Spengler, Nature 365 (1993), 170-175), 5' untranslated regions were excised and a HA epitope tag was added at the N terminus. Every construct was verified by sequencing.

The intracellular localization of hZAC was determined by immunocytochemistry. For immunocytochemistry, transfected cells were grown on glass cover slips, fixed, permeabilized, incubated with the anti-HA antibody and then with an anti-mouse FITC (Sigma, StLouis, MO). The nuclei of SaOs-2 cells expressing mZAC or hZAC were strongly labeled (Fig. 10A). No signal was detected neither in mock-transfected cells nor in non-permeabilized cells.

To ask whether hZAC was capable of transcriptional activity like many ZF proteins and p53, the transcriptional activation of the luciferase reporter, driven by a minimal promoter sensitive to the yeast transcription activator GAL4 was measured, after transfection of plasmids encoding hZAC or mZAC fused to the GAL4 DNA binding domain. For the GAL4 fusion protein, mZAC and hZAC coding sequences were inserted into the BamHI site of pSG424 (Blau, Mol. Cell. Biol. 16 (1996), 2044-2052). Different amounts of these expression vectors were transfected together with the reporter plasmid pEIBTATALUC (0.5 μ g), which contains five copies of the GAL4 DNA-binding site upstream of the the EIB-adenovirus derived TATAA box driving expression of the luciferase gene. The co-transfected plasmid pCH110 encoding the β -galactosidase gene driven by the SV40 promoter served to standardize luciferase values on transfection efficiency. Transfection of increasing amounts of the fusions containing either mZAC or hZAC induced a stepwise increase of the luciferase activity (Fig. 10B). The GAL4 DNA-binding domain (GAL1-147) alone was devoid of any transactivation activity (Fig. 10B). Fusions of the GAL4 DNA binding domain with the well characterized

transactivation domains of the transcription factors SP1 and CTF (Blau, Mol. Cell. Biol. 16 (1996), 2044-2052) served as positive controls (Fig. 10B).

Example 13: Expression of hZAC inhibited the growth of tumor cells

24h after transfection of hZAC, a reduced number of cells was observed, compared to mock transfected cells. Furthermore, after hZAC transfection, a substantial number of cells displayed signs of lost viability such as cellular shrinkage, blebbing, and condensed chromatin, as it was previously noted for p53 (Bates, Curr. Opin. Genet. Dev. 6 (1996), 12-19) and mZAC, see Example 6. To evaluate whether hZAC had the same antiproliferative properties as its mouse counterpart a colony formation assay was carried out. For colony formation assay, we cotransfected pRK5-PUR containing the puromycin-resistance gene under the control of a CMV promoter (0.2µg) with either mZAC (1 µg), hZAC (3.8 µg), or p53 (1µg) in sense and anti-sense orientations into 2 millions of SaOs-2 cells. The cells from three transfections were pooled and splitted in different plates. After 9 days of puromycin treatment, the clones were MTT stained and counted.

When either hZAC, mZAC or p53 was expressed, the puromycin-resistant clones were smaller than in the control experiments, mZAC or p53 strongly decreased the number of puromycin-resistant colonies (Fig. 11), whereas the antisense constructs had no effect. hZAC also inhibited cell growth although less strongly (Fig. 11).

Example 14: hZAC expression induced a G1 arrest

mZAC, like p53, inhibits tumor cell growth through induction of apoptosis and cell cycle arrest, see Examples 5 to 7. In order to determine whether hZAC retrieved the same mechanisms for the control of cell proliferation the cell cycle progression of SaOs-2 cells upon transient expression of hZAC was first investigated. SaOs-2 cells were transiently transfected with different amounts of plasmid encoding mZAC, hZAC or p53, together with pRK5-CD20 encoding the CD20 antigen which

was used as a marker for selection of the transfected cells. Propidium-iodide staining was performed as previously described (Brons, Cytometry 11 (1990), 837-844). Cell cycle distribution was determined with a FACScan flow cytometer (Beckton-Dickinson). 5000 events corresponding to the 5% CD20 most positive cells were analysed using Modfit software (Verity Software House, Inc). hZAC induced a G1 arrest with increased proportion of cells in G0-G1 and decreased in S (Fig. 12A). mZAC induced a G1 block (Fig. 12A). p53-transiently transfected SaOs-2 cells were strongly arrested in G1 (Fig. 12A) as previously reported (Chen, Genes Develop. 10 (1996), 2438-2451).

mZAC, hZAC and p53 expression levels were evaluated by Western blots performed with the same anti-HA antibody. Western blots were performed on total cell lysates (20µg protein) using anti-HA antibody (clone 12CA5, Boehringer Mannheim, France) and peroxylase-linked anti-mouse Ig (Amersham, France). p53 and mZAC reached comparable high levels of expression (Fig. 12B). Transfection of higher amounts of plasmid was required to detect hZAC (Fig. 12B). However even non-detectable levels of hZAC could induce a G1 arrest (see pRK-hZAC 125 and 250 ng, Fig. 12A, 12B).

Example 15: hZAC expression induced apoptosis

Apoptosis was investigated by measuring genomic DNA laddering. Soluble DNA was prepared as previously described (Hockenberry, Nature 348 (1990), 334-336) and fractionated on a 1.2% agarose gel.

Mock-transfected (lane 1) or control (pRK5-CAT) transfected cells (lane 2) did not show any sign of apoptosis (Fig. 13). hZAC expression induced apoptosis of SaOs-2 transfected cells (lanes 6,7,8) as noted for mZAC (lanes 3,4,5) and p53 (lane 9) as described in Example 6.

In summary, we have demonstrated that mZAC (Bop1) establishes a new class of TSG with its mammalian counterparts, e.g. rat and, in particular, human (hZAC). The identity of mZAC and hZAC genes was emphasized by the identical in vitro

properties of both proteins, for example the antiproliferative properties of these nuclear transcriptional activators. hZAC is the only human gene so far beside p53 able to regulate both apoptosis and cell cycle. Since these pathways are known to be central to the activity of the TSG p53 (Bates, Curr. Opin. Genet. Dev. 6 (1996), 12-19) and since hZAC maps to 6q24-q25, a chromosomal region frequently lost in human tumors, it is conclusive that hZAC is a candidate for a human TSG.

The present invention is not to be limited in scope by the specific embodiments described which are intended as single illustrations of individual aspects of the invention, and any nucleic acid molecules, proteins, constructs or antibodies which are functionally equivalent are within the scope of this invention. Indeed, various modification of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description and accompanying drawings. Such modifications are intended to fall within the scope of the appended claims.

SEQUENCE LISTING

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(ii) TITLE OF INVENTION: Nucleic acid molecules coding for mammalian
tumor suppressor proteins and methods for their isolation

(iii) NUMBER OF SEQUENCES: 17

(iv) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 2790 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(ix) FEATURE:

(A) NAME/KEY: CDS
(B) LOCATION: 542..2545

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

GAATTCGGGA GAGCAAGCGG GCATCTCCTG GGCGCCGTCA TGGCTGCTTA GGCTGCGCTG	60
CCTGCGGATC GCGGATCCGG GATCGGAGAT CTGACGGCGA CGCCTGAGTC CGGCTAGGGT	120
AGGTCTGGGT TGGAGTCTGT GCCTGCTTCC TTGGCGTGTG GTTGTTCTCT CTTGATTGCT	180
TCAGCGTGCC ATCGGCTTCG TATTTGCATA GGAGTCAGAG GAGTTAATCT TGTCTCCTCG	240
AAGATAGACT CTCATGGTTT ATGATCCATC TCTGTGAGAA GACTTTATTT GTCTGTCTCT	300

TCTCACAGGT TTGAGTCTTC AGACTTCTAC AGAACTCCAT AATATCTGCC TCACAGCTGG 360
 CTTTCCTGCT CTCACAGAAG ATACCCAGCT ATTGTGCTCT GGATCTCTCC TGGCTGCTAG 420
 GCTGTAGCGC TGCCTTTCTG GAGTCAGGCT GTAGTGA CTC CCCACCTTCT TTCTGTCTGG 480
 GCTTAAATGG CACAGCAGTT CCTCAGCACA TCTGAAGAAG AAAGTGTGAG AACCAAAGGC 540
 C ATG GCT CCA TTC CGC TGT CAA AAA TGT GGC AAG TCC TTC GTC ACC 586
 Met Ala Pro Phe Arg Cys Gln Lys Cys Gly Lys Ser Phe Val Thr
 1 5 10 15
 CTG GAG AAG TTC ACC ATT CAC AAT TAT TCC CAC TCC AGG GAG CGC CCA 634
 Leu Glu Lys Phe Thr Ile His Asn Tyr Ser His Ser Arg Glu Arg Pro
 20 25 30
 TTC AAG TGC TCG AAG GCT GAG TGT GGC AAA GCC TTC GTC TCC AAG TAT 682
 Phe Lys Cys Ser Lys Ala Glu Cys Gly Lys Ala Phe Val Ser Lys Tyr
 35 40 45
 AAG CTG ATG AGA CAC ATG GCC ACA CAC TCG CCA CAG AAG ATT CAC CAG 730
 Lys Leu Met Arg His Met Ala Thr His Ser Pro Gln Lys Ile His Gln
 50 55 60
 TGT ACT CAC TGT GAG AAG ACA TTC AAC CGG AAG GAC CAC CTG AAG AAC 778
 Cys Thr His Cys Glu Lys Thr Phe Asn Arg Lys Asp His Leu Lys Asn
 65 70 75
 CAC CTC CAG ACC CAC GAT CCC AAC AAG ATC TCC TAC GCG TGT GAC GAT 826
 His Leu Gln Thr His Asp Pro Asn Lys Ile Ser Tyr Ala Cys Asp Asp
 80 85 90 95
 TGC GGC AAG AAG TAC CAC ACC ATG CTG GGC TAC AAG AGG CAC CTG GCC 874
 Cys Gly Lys Lys Tyr His Thr Met Leu Gly Tyr Lys Arg His Leu Ala
 100 105 110
 CTG CAC TCG GCG AGC AAT GGC GAT CTC ACC TGT GGG GTG TGC ACC CTG 922
 Leu His Ser Ala Ser Asn Gly Asp Leu Thr Cys Gly Val Cys Thr Leu
 115 120 125
 GAG CTG GGG AGC ACC GAG GTC CTG CTG GAC CAC CTC AAG TCT CAC GCG 970
 Glu Leu Gly Ser Thr Glu Val Leu Leu Asp His Leu Lys Ser His Ala
 130 135 140
 GAA GAA AAG GCC AAC CAG GCA CCC AGG GAG AAG AAA TAC CAG TGC GAC 1018
 Glu Glu Lys Ala Asn Gln Ala Pro Arg Glu Lys Lys Tyr Gln Cys Asp
 145 150 155
 CAC TGT GAT AGA TGC TTC TAC ACC CGG AAA GAT GTG CGT CGC CAC CTG 1066
 His Cys Asp Arg Cys Phe Tyr Thr Arg Lys Asp Val Arg Arg His Leu
 160 165 170 175
 GTG GTC CAC ACA GGA TGC AAG GAC TTC CTG TGT CAG TTC TGT GCC CAG 1114
 Val Val His Thr Gly Cys Lys Asp Phe Leu Cys Gln Phe Cys Ala Gln
 180 185 190
 AGA TTT GGG CGC AAA GAC CAC CTC ACT CGT CAC ACC AAG AAG ACC CAC 1162
 Arg Phe Gly Arg Lys Asp His Leu Thr Arg His Thr Lys Lys Thr His
 195 200 205

664730"0234360

TCC Ser	CAG Gln	GAG Glu 210	CTG Leu	ATG Met	CAA Gln	GAG Glu	AAT Asn 215	ATG Met	CAG Gln	GCA Ala	GGA Gly	GAT Asp 220	TAC Tyr	CAG Gln	AGC Ser	1210
AAT Asn 225	TTC Phe	CAA Gln	CTC Leu	ATT Ile	GCG Ala	CCT Pro 230	TCA Ser	ACT Thr	TCG Ser	TTC Phe	CAG Gln 235	ATA Ile	AAG Lys	GTT Val	GAT Asp	1258
CCC Pro 240	ATG Met	CCT Pro	CCT Pro	TTC Phe	CAG Gln 245	CTA Leu	GGA Gly	GCG Ala	GCT Ala	CCC Pro 250	GAG Glu	AAC Asn	GGG Gly	CTT Leu	GAT Asp 255	1306
GGT Gly	GGC Gly	TTG Leu	CCA Pro	CCC Pro 260	GAG Glu	GTT Val	CAT His	GGT Gly	CTA Leu 265	GTG Val	CTT Leu	GCT Ala	GCC Ala 270	CCA Pro	GAA Glu	1354
GAA Glu	GCT Ala	CCC Pro	CAA Gln 275	CCC Pro	ATG Met	CCG Pro	CCC Pro	TTG Leu 280	GAG Glu	CCT Pro	TTG Leu	GAG Glu	CCT Pro 285	TTG Leu	GAG Glu	1402
CCT Pro	TTG Leu	GAG Glu 290	CCT Pro	TTG Leu	GAG Glu	CCG Pro	ATG Met 295	CAG Gln	TCT Ser	TTG Leu	GAG Glu	CCT Pro 300	TTG Leu	CAG Gln	CCT Pro	1450
TTG Leu 305	GAG Glu	CCG Pro	ATG Met	CAG Gln	CCT Pro	TTG Leu 310	GAG Glu	CCA Pro	ATG Met	CAG Gln 315	CCT Pro	TTG Leu	GAG Glu	CCG Pro	ATG Met	1498
CAG Gln 320	CCT Pro	TTA Leu	GAG Glu	CCT Pro	TTG Leu 325	GAG Glu	CCT Pro	CTG Leu	GAG Glu	CCG Pro 330	ATG Met	CAG Gln	CCT Pro	TTG Leu	GAG Glu 335	1546
CCG Pro	ATG Met	CAG Gln	CCT Pro	TTG Leu 340	GAG Glu	CCT Pro	ATG Met	CAG Gln	CCA Pro 345	ATG Met	CTG Leu	CCA Pro	ATG Met	CAG Gln 350	CCA Pro	1594
ATG Met	CAG Gln	CCA Pro	ATG Met 355	CAG Gln	CCA Pro	ATG Met	CAG Gln	CCA Pro 360	ATG Met	CTG Leu	CCA Pro	ATG Met	CAG Gln 365	CCA Pro	ATG Met	1642
CTG Leu	CCA Pro	ATG Met 370	CAG Gln	CCA Pro	ATG Met	CAG Gln	CCA Pro 375	ATG Met	CAG Gln	CCA Pro	ATG Met	CTG Leu 380	CCA Pro	ATG Met	CCA Pro	1690
GAG Glu 385	CCG Pro	TCT Ser	TTC Phe	ACT Thr	CTG Leu	CAC His 390	CCT Pro	GGC Gly	GTA Val	GTT Val	CCC Pro 395	ACC Thr	TCT Ser	CCT Pro	CCC Pro	1738
CCA Pro 400	ATT Ile	ATT Ile	CTT Leu	CAG Gln 405	GAG Glu	CAT His	AAG Lys	TAT Tyr	AAT Asn	CCT Pro 410	GTT Val	CCT Pro	ACC Thr	TCA Ser	TAT Tyr 415	1786
GCC Ala	CCA Pro	TTT Phe	GTA Val	GGC Gly 420	ATG Met	CCC Pro	GTC Val	AAA Lys	GCA Ala 425	GAT Asp	GGC Gly	AAG Lys	GCC Ala	TTT Phe 430	TGC Cys	1834
AAC Asn	GTG Val	GGT Gly	TTC Phe	TTT Phe	GAG Glu	GAA Glu	TTT Phe	CCT Pro	CTG Leu	CAA Gln	GAG Glu	CCT Pro	CAG Gln	GCG Ala	CCT Pro	1882

435																440																445																
CTC	AAG	TTC	AAC	CCA	TGT	TTT	GAG	ATG	CCT	ATG	GAG	GGG	TTT	GGG	AAA	1930																																
Leu	Lys	Phe	Asn	Pro	Cys	Phe	Glu	Met	Pro	Met	Glu	Gly	Phe	Gly	Lys																																	
450				455				460																																								
GTC	ACC	CTG	TCC	AAA	GAG	CTG	CTG	GTA	GAT	GCT	GTG	AAT	ATA	GCC	ATT	1978																																
Val	Thr	Leu	Ser	Lys	Glu	Leu	Leu	Val	Asp	Ala	Val	Asn	Ile	Ala	Ile																																	
465				470				475																																								
CCT	GCC	TCT	CTG	GAG	ATT	TCC	TCC	CTA	TTG	GGG	TTT	TGG	CAG	CTC	CCC	2026																																
Pro	Ala	Ser	Leu	Glu	Ile	Ser	Ser	Leu	Leu	Gly	Phe	Trp	Gln	Leu	Pro																																	
480				485				490				495																																				
CCT	CCT	ACT	CCC	CAG	AAT	GGC	TTT	GTG	AAT	AGC	ACC	ATC	CCT	GTG	GGG	2074																																
Pro	Pro	Thr	Pro	Gln	Asn	Gly	Phe	Val	Asn	Ser	Thr	Ile	Pro	Val	Gly																																	
500				505				510																																								
CCT	GGG	GAG	CCA	CTG	CCC	CAT	AGG	ATA	ACC	TGT	CTG	GCG	CAG	CAG	CAG	2122																																
Pro	Gly	Glu	Pro	Leu	Pro	His	Arg	Ile	Thr	Cys	Leu	Ala	Gln	Gln	Gln																																	
515				520				525																																								
CCA	CCG	CCA	CTG	CCG	CCG	CCA	CCA	CCG	CTG	CCA	CTG	CCA	CAG	CCA	CTG	2170																																
Pro	Pro	Pro	Leu	Pro	Pro	Pro	Pro	Pro	Leu	Pro	Leu	Pro	Gln	Pro	Leu																																	
530				535				540																																								
CCA	GTG	CCA	CAG	CCA	CTA	CCA	CAG	CCA	CAG	ATG	CAG	CCA	CAG	TTT	CAG	2218																																
Pro	Val	Pro	Gln	Pro	Leu	Pro	Gln	Pro	Gln	Met	Gln	Pro	Gln	Phe	Gln																																	
545				550				555																																								
TTG	CAG	ATC	CAG	CCC	CAG	ATG	CAG	CTA	CCA	CAG	CTG	CTG	CCG	CAA	CTG	2266																																
Leu	Gln	Ile	Gln	Pro	Gln	Met	Gln	Leu	Pro	Gln	Leu	Leu	Pro	Gln	Leu																																	
560				565				570				575																																				
CAA	CCT	CAG	CAG	CAG	CCT	GAT	CCT	GAG	CCA	GAG	CCA	GAG	CCA	GAG	CCA	2314																																
Gln	Pro	Gln	Gln	Gln	Pro	Asp	Pro	Glu	Pro	Glu	Pro	Glu	Pro	Glu	Pro																																	
580				585				590																																								
GAG	CCA	GAG	CCA	GAG	CCA	GAG	CCG	GAA	CCG	GAA	CCG	GAG	CCA	GAG	CCA	2362																																
Glu	Pro	Glu	Pro	Glu	Pro	Glu	Pro	Glu	Pro	Glu	Pro	Glu	Pro	Glu	Pro																																	
595				600				605																																								
GAG	CCA	GAA	CCA	GAG	CCA	GAG	GAA	GAA	CAG	GAA	GAG	GCA	GAA	GAA	GAG	2410																																
Glu	Pro	Glu	Pro	Glu	Pro	Glu	Glu	Glu	Gln	Glu	Glu	Ala	Glu	Glu	Glu																																	
610				615				620																																								
GCA	GAG	GAA	GGA	GCA	GAG	GAA	GGA	GCA	GAA	CCA	GAG	GCA	CAG	GCA	GAA	2458																																
Ala	Glu	Glu	Gly	Ala	Glu	Glu	Gly	Ala	Glu	Pro	Glu	Ala	Gln	Ala	Glu																																	
625				630				635																																								
GAA	GAG	GAA	GAG	GAA	GAG	GAA	GCG	GAA	GAG	CCA	CAG	CCA	GAA	GAA	GCC	2506																																
Glu	Glu	Glu	Glu	Glu	Glu	Glu	Ala	Glu	Glu	Pro	Gln	Pro	Glu	Glu	Ala																																	
640				645				650				655																																				
CAA	ATA	GCA	GGA	CTC	GTC	TAT	AAG	AAA	TGG	ACA	GTT	TAG	TTCCTCTTCT			2555																																
Gln	Ile	Ala	Gly	Leu	Val	Tyr	Lys	Lys	Trp	Thr	Val	*																																				
660				665																																												
TGTTAGCTTA CTCTGTAGTT TCTTCTTCTT GTTGCCCATTT GTGTAGCTTT ATAGAGTGTG																2615																																

(2) INFORMATION FOR SEO ID NO: 2:

(A) LENGTH: 668 amino acids

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

Met 1	Ala	Pro	Phe	Arg 5	Cys	Gln	Lys	Cys	Gly 10	Lys	Ser	Phe	Val 15	Thr	Leu
Glu	Lys	Phe	Thr 20	Ile	His	Asn	Tyr	Ser 25	His	Ser	Arg	Glu	Arg 30	Pro	Phe
Lys	Cys	Ser 35	Lys	Ala	Glu	Cys	Gly 40	Lys	Ala	Phe	Val	Ser 45	Lys	Tyr	Lys
Leu	Met 50	Arg	His	Met	Ala	Thr 55	His	Ser	Pro	Gln	Lys 60	Ile	His	Gln	Cys
Thr 65	His	Cys	Glu	Lys	Thr 70	Phe	Asn	Arg	Lys	Asp 75	His	Leu	Lys	Asn	His 80
Leu	Gln	Thr	His	Asp 85	Pro	Asn	Lys	Ile	Ser 90	Tyr	Ala	Cys	Asp	Asp 95	Cys
Gly	Lys	Lys	Tyr 100	His	Thr	Met	Leu	Gly 105	Tyr	Lys	Arg	His	Leu 110	Ala	Leu
His	Ser	Ala 115	Ser	Asn	Gly	Asp	Leu 120	Thr	Cys	Gly	Val	Cys 125	Thr	Leu	Glu
Leu	Gly 130	Ser	Thr	Glu	Val	Leu 135	Leu	Asp	His	Leu	Lys 140	Ser	His	Ala	Glu
Glu 145	Lys	Ala	Asn	Gln	Ala 150	Pro	Arg	Glu	Lys	Lys 155	Tyr	Gln	Cys	Asp	His 160
Cys	Asp	Arg	Cys	Phe 165	Tyr	Thr	Arg	Lys	Asp 170	Val	Arg	Arg	His	Leu 175	Val
Val	His	Thr	Gly 180	Cys	Lys	Asp	Phe	Leu 185	Cys	Gln	Phe	Cys	Ala 190	Gln	Arg
Phe	Gly	Arg 195	Lys	Asp	His	Leu	Thr 200	Arg	His	Thr	Lys	Lys 205	Thr	His	Ser
Gln	Glu	Leu	Met	Gln	Glu	Asn	Met	Gln	Ala	Gly	Asp	Tyr	Gln	Ser	Asn

210					215					220					
Phe	Gln	Leu	Ile	Ala	Pro	Ser	Thr	Ser	Phe	Gln	Ile	Lys	Val	Asp	Pro
225					230					235					240
Met	Pro	Pro	Phe	Gln	Leu	Gly	Ala	Ala	Pro	Glu	Asn	Gly	Leu	Asp	Gly
				245					250					255	
Gly	Leu	Pro	Pro	Glu	Val	His	Gly	Leu	Val	Leu	Ala	Ala	Pro	Glu	Glu
				260				265					270		
Ala	Pro	Gln	Pro	Met	Pro	Pro	Leu	Glu	Pro	Leu	Glu	Pro	Leu	Glu	Pro
		275					280					285			
Leu	Glu	Pro	Leu	Glu	Pro	Met	Gln	Ser	Leu	Glu	Pro	Leu	Gln	Pro	Leu
	290					295					300				
Glu	Pro	Met	Gln	Pro	Leu	Glu	Pro	Met	Gln	Pro	Leu	Glu	Pro	Met	Gln
305				310					315					320	
Pro	Leu	Glu	Pro	Leu	Glu	Pro	Leu	Glu	Pro	Met	Gln	Pro	Leu	Glu	Pro
				325					330					335	
Met	Gln	Pro	Leu	Glu	Pro	Met	Gln	Pro	Met	Leu	Pro	Met	Gln	Pro	Met
			340				345						350		
Gln	Pro	Met	Gln	Pro	Met	Gln	Pro	Met	Leu	Pro	Met	Gln	Pro	Met	Leu
		355					360					365			
Pro	Met	Gln	Pro	Met	Gln	Pro	Met	Gln	Pro	Met	Leu	Pro	Met	Pro	Glu
	370					375				380					
Pro	Ser	Phe	Thr	Leu	His	Pro	Gly	Val	Val	Pro	Thr	Ser	Pro	Pro	Pro
385					390					395					400
Ile	Ile	Leu	Gln	Glu	His	Lys	Tyr	Asn	Pro	Val	Pro	Thr	Ser	Tyr	Ala
				405					410					415	
Pro	Phe	Val	Gly	Met	Pro	Val	Lys	Ala	Asp	Gly	Lys	Ala	Phe	Cys	Asn
			420					425					430		
Val	Gly	Phe	Phe	Glu	Glu	Phe	Pro	Leu	Gln	Glu	Pro	Gln	Ala	Pro	Leu
		435					440					445			
Lys	Phe	Asn	Pro	Cys	Phe	Glu	Met	Pro	Met	Glu	Gly	Phe	Gly	Lys	Val
	450					455					460				
Thr	Leu	Ser	Lys	Glu	Leu	Leu	Val	Asp	Ala	Val	Asn	Ile	Ala	Ile	Pro
465					470					475					480
Ala	Ser	Leu	Glu	Ile	Ser	Ser	Leu	Leu	Gly	Phe	Trp	Gln	Leu	Pro	Pro
				485					490					495	
Pro	Thr	Pro	Gln	Asn	Gly	Phe	Val	Asn	Ser	Thr	Ile	Pro	Val	Gly	Pro
			500					505					510		
Gly	Glu	Pro	Leu	Pro	His	Arg	Ile	Thr	Cys	Leu	Ala	Gln	Gln	Gln	Pro
		515					520					525			

000100 020000

Pro Pro Leu Pro Pro Pro Pro Pro Leu Pro Leu Pro Gln Pro Leu Pro
 530 535 540
 Val Pro Gln Pro Leu Pro Gln Pro Gln Met Gln Pro Gln Phe Gln Leu
 545 550 555 560
 Gln Ile Gln Pro Gln Met Gln Leu Pro Gln Leu Leu Pro Gln Leu Gln
 565 570 575
 Pro Gln Gln Gln Pro Asp Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu
 580 585 590
 Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu Pro Glu
 595 600 605
 Pro Glu Pro Glu Pro Glu Glu Glu Gln Glu Glu Ala Glu Glu Glu Ala
 610 615 620
 Glu Glu Gly Ala Glu Glu Gly Ala Glu Pro Glu Ala Gln Ala Glu Glu
 625 630 635 640
 Glu Glu Glu Glu Glu Glu Ala Glu Glu Pro Gln Pro Glu Glu Ala Gln
 645 650 655
 Ile Ala Gly Leu Val Tyr Lys Lys Trp Thr Val *
 660 665

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid

- (A) DESCRIPTION: /desc = "oligonucleotide"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

ATGTCTCGAG GCCTTTGCGG CCGCTATANN NNNNNN

36

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

9

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

```
(ix) FEATURE:
      (A) NAME/KEY: -
      (B) LOCATION:3
      (D) OTHER INFORMATION:/note= "N at position 3 is A or G."
```

9

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 9 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(iii) HYPOTHETICAL: YES

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 35 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 10:

35

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 11:

30

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 25 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 12:

25

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 13:

33

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 18 base pairs

(B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 14:

ATAGCAGTGA GTGCTGTG

18

(2) INFORMATION FOR SEQ ID NO: 15:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 18 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 15:

GTTTCTTTTC AGGGACTC

18

(2) INFORMATION FOR SEQ ID NO: 16:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 2334 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: CDS
 (B) LOCATION:803..2192

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 16:

CGGTTCTTTC AATTCAGAAT TTGTTTTAGG TTCTGTTATT GCATAGATTT GCATACCTGT	60
TTTATGGTAT TTTAATACTG TTGGTTTTAA AAAATACCAT TTCCTCTGAG TGCTGTTCTG	120
AATATATTAT GTAAGCAATT TTGTGTGTTT TTTTTTTTCC ACTTGCATAA AGCAGGGGAA	180
AAGTTGAGAG TTTTCTTAA TCCAGTCCCA AGTAGGACAA AGGATATGAG TGTTTAAAGA	240
TCATCTATTA AAATGCATGA AAAAACACTA GAAAATCTCC TGTGCACATC GCCAGTCGTG	300
TGTGTGCTCT AGAAGTGAAG TTCAGGGGGT AACATAATGG AGGAATGTTT TCCTAGCTTC	360
ATTCCCTGAC GATGTACAAG GTCTCTTCTC ACAGGTTTGA ATCTTCAGAC AAACCTCTGG	420

GAGGACTGGG	AGGACTCGGT	CCCTGCCTCG	CAGCAGATGT	TCCCTGTCAC	TCAGTAGCCA	480
ATCCGGGGGA	CCCAGGACAT	GCCCCAGCTA	TAGTGATGCA	GATTACCTTT	CTGGTCCTGA	540
ATCGCACCTG	TGCCTCGAGA	CTTTCTCCCC	TCAGCTTGAG	ACTGCATGTA	AACTGGGATG	600
TGTGAAAGCA	GGAAGCAAAG	CTAGTGACAG	CTGAGAGGTC	CATGTCTGGG	TAGAACCAGG	660
CCCACGATGC	TGCCTCTCCC	GTGGTCTGGA	GTTCAGCTGC	AGGGACTCTG	CTGATTGGCC	720
CAGCACCATC	GTTCTGTTTG	TGCTTAAATG	GCACAGCATT	TGGTCAGCAC	ATCTGAAAAG	780
GAAGGTGTGA	GAAGCAAAGC	CC ATG GCC ACG TTC CCC TGC CAG TTA TGT GGC				832
		Met Ala Thr Phe Pro Cys Gln Leu Cys Gly				
		1 5 10				
AAG ACG TTC CTC ACC CTG GAG AAG TTC ACG ATT CAC AAT TAT TCC CAC						880
Lys Thr Phe Leu Thr Leu Glu Lys Phe Thr Ile His Asn Tyr Ser His						
		15 20 25				
TCC AGG GAG CGG CCG TAC AAG TGT GTG CAG CCT GAC TGT GGC AAA GCC						928
Ser Arg Glu Arg Pro Tyr Lys Cys Val Gln Pro Asp Cys Gly Lys Ala						
		30 35 40				
TTT GTT TCC AGA TAT AAA TTG ATG AGG CAT ATG GCT ACC CAT TCT CCC						976
Phe Val Ser Arg Tyr Lys Leu Met Arg His Met Ala Thr His Ser Pro						
		45 50 55				
CAG AAA TCT CAC CAG TGT GCT CAC TGT GAG AAG ACG TTC AAC CGG AAA						1024
Gln Lys Ser His Gln Cys Ala His Cys Glu Lys Thr Phe Asn Arg Lys						
		60 65 70				
GAC CAC CTG AAA AAC CAC CTC CAG ACC CAC GAC CCC AAC AAA ATG GCC						1072
Asp His Leu Lys Asn His Leu Gln Thr His Asp Pro Asn Lys Met Ala						
		75 80 85 90				
TTT GGG TGT GAG GAG TGT GGG AAG AAG TAC AAC ACC ATG CTG GGC TAT						1120
Phe Gly Cys Glu Glu Cys Gly Lys Lys Tyr Asn Thr Met Leu Gly Tyr						
		95 100 105				
AAG AGG CAC CTG GCC CTC CAT GCG GCC AGC AGT GGG GAC CTC ACC TGT						1168
Lys Arg His Leu Ala Leu His Ala Ala Ser Ser Gly Asp Leu Thr Cys						
		110 115 120				
GGG GTC TGT GCC CTG GAG CTA GGG AGC ACC GAG GTG CTA CTG GAC CAC						1216
Gly Val Cys Ala Leu Glu Leu Gly Ser Thr Glu Val Leu Leu Asp His						
		125 130 135				
CTC AAA GCC CAT GCG GAA GAG AAG CCC CCT AGC GGA ACC AAG GAA AAG						1264
Leu Lys Ala His Ala Glu Glu Lys Pro Pro Ser Gly Thr Lys Glu Lys						
		140 145 150				
AAG CAC CAG TGC GAC CAC TGT GAA AGA TGC TTC TAC ACC CGG AAG GAT						1312
Lys His Gln Cys Asp His Cys Glu Arg Cys Phe Tyr Thr Arg Lys Asp						
		155 160 165 170				

GTG	CGA	CGC	CAC	CTG	GTG	GTC	CAC	ACA	GGA	TGC	AAG	GAC	TTC	CTG	TGC	1360
Val	Arg	Arg	His	Leu	Val	Val	His	Thr	Gly	Cys	Lys	Asp	Phe	Leu	Cys	
			175						180					185		
CAG	TTC	TGT	GCC	CAG	AGA	TTT	GGG	CGC	AAG	GAT	CAC	CTC	ACC	CGG	CAT	1408
Gln	Phe	Cys	Ala	Gln	Arg	Phe	Gly	Arg	Lys	Asp	His	Leu	Thr	Arg	His	
			190					195					200			
ACC	AAG	AAG	ACC	CAC	TCA	CAG	GAG	CTG	ATG	AAA	GAG	AGC	TTG	CAG	ACC	1456
Thr	Lys	Lys	Thr	His	Ser	Gln	Glu	Leu	Met	Lys	Glu	Ser	Leu	Gln	Thr	
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Gly	Asp	Leu	Leu	Ser	Thr	Phe	His	Thr	Ile	Ser	Pro	Ser	Phe	Gln	Leu	
	220					225					230					
AAG	GCT	GCT	GCC	TTG	CCT	CCT	TTC	CCT	TTA	GGA	GCT	TCT	GCC	CAG	AAC	1552
Lys	Ala	Ala	Ala	Leu	Pro	Pro	Phe	Pro	Leu	Gly	Ala	Ser	Ala	Gln	Asn	
235				240						245					250	
GGG	CTT	GCA	AGT	AGC	TTG	CCA	GCT	GAG	GTC	CAT	AGC	CTC	ACC	CTC	AGT	1600
Gly	Leu	Ala	Ser	Ser	Leu	Pro	Ala	Glu	Val	His	Ser	Leu	Thr	Leu	Ser	
			255						260					265		
CCC	CCA	GAA	CAA	GCC	GCC	CAG	CCT	ATG	CAG	CCG	CTG	CCA	GAG	TCC	CTG	1648
Pro	Pro	Glu	Gln	Ala	Ala	Gln	Pro	Met	Gln	Pro	Leu	Pro	Glu	Ser	Leu	
			270					275					280			
GCC	TCC	CTC	CAC	CCC	TCG	GTA	TCC	CCT	GGC	TCT	CCT	CCG	CCA	CCC	CTT	1696
Ala	Ser	Leu	His	Pro	Ser	Val	Ser	Pro	Gly	Ser	Pro	Pro	Pro	Pro	Leu	
		285					290					295				
CCC	AAT	CAC	AAG	TAC	AAC	ACC	ACT	TCT	ACC	TCA	TAC	TCC	CCA	CTT	GCA	1744
Pro	Asn	His	Lys	Tyr	Asn	Thr	Thr	Ser	Thr	Ser	Tyr	Ser	Pro	Leu	Ala	
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315				320					325						330	
TTT	GAG	GAC	TTG	CCT	CTG	CAA	GAG	CCT	CAG	TCA	CCT	CAA	AAG	CTC	AAC	1840
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Pro	Gly	Phe	Asp	Leu	Ala	Lys	Gly	Asn	Ala	Gly	Lys	Val	Asn	Leu	Pro	
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Lys	Glu	Leu	Pro	Ala	Asp	Ala	Val	Asn	Leu	Thr	Ile	Pro	Ala	Ser	Leu	
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GAC	CTG	TCC	CCC	CTG	TTG	GGC	TTC	TGG	CAG	CTG	CCC	CCT	CCT	GCT		

380	385	390	
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Gln Asn Thr Phe Gly Asn Ser Thr Leu Ala Leu Gly Pro Gly Glu Ser			
395	400	405	410
TTG CCC CAC AGG TTA AGC TGT CTG GGG CAG CAG CAG CAA GAA CCC CCA			2080
Leu Pro His Arg Leu Ser Cys Leu Gly Gln Gln Gln Gln Glu Pro Pro			
	415	420	425
CTT GCC ATG GGC ACT GTG AGC CTG GGC CAG CTC CCC CTG CCC CCC ATC			2128
Leu Ala Met Gly Thr Val Ser Leu Gly Gln Leu Pro Leu Pro Pro Ile			
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CCT CAT GTG TTC TCA GCT GGC ACT GGC TCT GCC ATC CTG CCT CAT TTC			2176
Pro His Val Phe Ser Ala Gly Thr Gly Ser Ala Ile Leu Pro His Phe			
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CAT CAT GCA TTC AGA T AATTGATTTT TAAAGTGTAT TTTTCGTATT			2222
His His Ala Phe Arg			
	460		
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(2) INFORMATION FOR SEQ ID NO: 17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 463 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 17:

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Glu	Lys	Phe	Thr	Ile	His	Asn	Tyr	Ser	His	Ser	Arg	Glu	Arg	Pro	Tyr
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Lys	Cys	Val	Gln	Pro	Asp	Cys	Gly	Lys	Ala	Phe	Val	Ser	Arg	Tyr	Lys
		35					40					45			
Leu	Met	Arg	His	Met	Ala	Thr	His	Ser	Pro	Gln	Lys	Ser	His	Gln	Cys
	50					55					60				
Ala	His	Cys	Glu	Lys	Thr	Phe	Asn	Arg	Lys	Asp	His	Leu	Lys	Asn	His
65					70					75				80	
Leu	Gln	Thr	His	Asp	Pro	Asn	Lys	Met	Ala	Phe	Gly	Cys	Glu	Glu	Cys
				85					90					95	

Gly	Lys	Lys	Tyr	Asn	Thr	Met	Leu	Gly	Tyr	Lys	Arg	His	Leu	Ala	Leu	
			100						105			110				
His	Ala	Ala	Ser	Ser	Gly	Asp	Leu	Thr	Cys	Gly	Val	Cys	Ala	Leu	Glu	
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Leu	Gly	Ser	Thr	Glu	Val	Leu	Leu	Asp	His	Leu	Lys	Ala	His	Ala	Glu	
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Cys	Glu	Arg	Cys	Phe	Tyr	Thr	Arg	Lys	Asp	Val	Arg	Arg	His	Leu	Val	
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Val	His	Thr	Gly	Cys	Lys	Asp	Phe	Leu	Cys	Gln	Phe	Cys	Ala	Gln	Arg	
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Phe	Gly	Arg	Lys	Asp	His	Leu	Thr	Arg	His	Thr	Lys	Lys	Thr	His	Ser	
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Gln	Glu	Leu	Met	Lys	Glu	Ser	Leu	Gln	Thr	Gly	Asp	Leu	Leu	Ser	Thr	
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Phe	His	Thr	Ile	Ser	Pro	Ser	Phe	Gln	Leu	Lys	Ala	Ala	Ala	Leu	Pro	
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Pro	Phe	Pro	Leu	Gly	Ala	Ser	Ala	Gln	Asn	Gly	Leu	Ala	Ser	Ser	Leu	
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Gln	Pro	Met	Gln	Pro	Leu	Pro	Glu	Ser	Leu	Ala	Ser	Leu	His	Pro	Ser	
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			305						310			315			320	
Asp	Thr	Lys	Gly	Phe	Cys	Asn	Ile	Ser	Leu	Phe	Glu	Asp	Leu	Pro	Leu	
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Gln	Glu	Pro	Gln	Ser	Pro	Gln	Lys	Leu	Asn	Pro	Gly	Phe	Asp	Leu	Ala	
			340						345			350				
Lys	Gly	Asn	Ala	Gly	Lys	Val	Asn	Leu	Pro	Lys	Glu	Leu	Pro	Ala	Asp	
			355						360			365				
Ala	Val	Asn	Leu	Thr	Ile	Pro	Ala	Ser	Leu	Asp	Leu	Ser	Pro	Leu	Leu	
			370						375			380				

Gly	Phe	Trp	Gln	Leu	Pro	Pro	Pro	Ala	Thr	Gln	Asn	Thr	Phe	Gly	Asn
385					390					395					400
Ser	Thr	Leu	Ala	Leu	Gly	Pro	Gly	Glu	Ser	Leu	Pro	His	Arg	Leu	Ser
				405					410					415	
Cys	Leu	Gly	Gln	Gln	Gln	Gln	Glu	Pro	Pro	Leu	Ala	Met	Gly	Thr	Val
			420					425					430		
Ser	Leu	Gly	Gln	Leu	Pro	Leu	Pro	Pro	Ile	Pro	His	Val	Phe	Ser	Ala
		435					440					445			
Gly	Thr	Gly	Ser	Ala	Ile	Leu	Pro	His	Phe	His	His	Ala	Phe	Arg	
	450					455					460				

CLAIMS

1. A nucleic acid molecule encoding a protein having the biological activity of a tumor suppressor selected from the group consisting of:
 - (a) nucleic acid molecules coding for a polypeptide comprising the amino acid sequence given in SEQ ID NO.2;
 - (b) nucleic acid molecules comprising the nucleotide sequence given in SEQ ID NO.1;
 - (c) nucleic acid molecules hybridizing to a nucleic acid molecule as defined in (a) or (b); and
 - (d) nucleic acid molecules, the nucleotide sequence of which is degenerate as a result of the genetic code to a nucleotide sequence of a nucleic acid molecule as defined in (a), (b) or (c).

2. A method for the identification and cloning of nucleic acid molecules encoding a protein having the biological activity of a tumor suppressor comprising the steps of:
 - (i) transfecting mammalian cells with
 - (a) a first vector comprising a scorable reporter gene operatively linked to regulatory elements comprising at least one cAMP responsive element so located relative to said reporter gene to permit cAMP inducible expression thereof; and
 - (b) pools of expression vectors comprising nucleic acid molecules linked to regulatory elements allowing expression in the mammalian cells;
 - (ii) cultivating the transfected cells under conditions which permit expression of the nucleic acid molecules present in the vectors;
 - (iii) identifying those vector pools which lead after transfection to expression of said reporter gene in the mammalian cells;

- (iv) optionally subdividing the vector pool(s) identified in step (iii) and repeating step (i) to (iii); and
 - (v) isolating from the so-identified vector pool(s) the nucleic acid molecule present in the vector(s) and testing its product for tumor suppressor activity.
3. The method of claim 2, wherein in step (ii) a ligand of a receptor which is capable of increasing the level of intracellular cAMP is added to the culture medium.
 4. The method of claim 3, wherein the ligand is the peptide PACAP.
 5. The method of any one of claims 2 to 4, wherein the mammalian cells are LLC-PK1 cells (ATCC CC101) or Saos-2 cells (ATCC HTB 85).
 6. The method of any one of claims 2 to 5, wherein the cAMP responsive element is derived from a corticotropin releasing hormone gene.
 7. The method of any one of claims 2 to 6, wherein the regulatory elements controlling the reporter gene are derived from MMTV.
 8. The method of any one of claims 2 to 7, wherein the reporter gene codes for a luciferase.
 9. The method of any one of claims 2 to 8, wherein the nucleic acid molecules present in the vectors of the vector pool are cDNAs.
 10. The method of claim 9, wherein the cDNA is prepared from RNA obtained from mammalian, bacterial, fungal or plant cells or viruses.

11. A nucleic acid molecule obtainable by a method of any one of claims 2 to 10 which encodes a protein having tumor suppressor activity.
12. A nucleic acid molecule which hybridizes to a nucleic acid molecule of claim 1 or claim 11 and which encodes a mutated version of a protein as defined in claim 1 and 11 which has lost its tumor suppressor activity.
13. The nucleic acid molecule of claim 1, 11 or 12 which is DNA.
14. The nucleic acid molecule of claim 13 which is cDNA.
15. The nucleic acid molecule of claim 1 or of any one of claims 11 to 14, which is derived from a mammal.
16. The nucleic acid molecule of claim 15, wherein the mammal is human or mouse.
17. The nucleic acid molecule of claim 16, wherein the nucleic acid molecule comprises a nucleotide sequence encoding the amino acid sequence given in SEQ ID NO. 17 or the nucleotide sequence given in SEQ ID NO. 16.
18. A nucleic acid molecule of at least 15 nucleotides in length hybridizing specifically with a nucleic acid molecule of claim 1 or with a nucleic acid molecule of any one of claims 11 to 17 or to a complementary strand thereof.
19. A vector comprising a nucleic acid molecule of claim 1 or of any one of claims 11 to 17.

20. The vector of claim 19, wherein the nucleic acid molecule is operatively linked to regulatory elements permitting expression in prokaryotic and/or eukaryotic host cells.
21. A host cell comprising a vector of claim 19 or 20.
22. The host cell of claim 21, which is a bacterial, fungal, plant or animal cell.
23. The host cell of claim 22, which is a mammalian cell.
24. Method for the production of a polypeptide having the biological activity of a tumor suppressor comprising culturing a host cell of claim 22 or 23 under conditions allowing the expression of the polypeptide and recovering the produced polypeptide from the culture.
25. A polypeptide encoded by a nucleic acid molecule of claim 1 or of any one of claims 11 to 17 or produced by a method according to claim 24.
26. An antibody specifically recognizing a polypeptide of claim 25.
27. A pharmaceutical composition comprising a nucleic acid molecule of claim 1 or of any one of claims 11 to 17, or a nucleic acid molecule which is complementary to such a nucleic acid molecule, a nucleic acid molecule of claim 18, a vector of claim 19 or 20, a polypeptide of claim 25 and/or an antibody of claim 26, and optionally a pharmaceutically acceptable carrier.
28. A diagnostic composition comprising a nucleic acid molecule of claim 1, or of any one of claims 11 to 17 or a nucleic acid molecule which is complementary to such a nucleic acid molecule, a nucleic acid molecule

of claim 18, a vector of claim 19 or 20, a polypeptide of claim 25 and/or an antibody of claim 26, and optionally suitable means for detection.

29. A method for treating of a tumor comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.
30. A method for preventing of a tumor comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.
31. A method for delaying the reoccurrence of a tumor comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.
32. The method of 29, 30 or 31 wherein the tumor is benign or malign and most preferably derived from endocrine or neuronal tissues, i. e. breast, lung, colon, intestine, stomach, prostate, testis, ovary, thyroid, pancreas.
33. A method for treating of neuronal disorders comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.
34. A method for preventing neuronal disorders comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.
35. A method for delaying the reoccurrence of neuronal disorders comprising administering to the subject the pharmaceutical composition of claim 27 in an effective dose.
36. A method for detecting expression of a tumor suppressor by detecting the presence of mRNA coding for a tumor suppressor which comprises

- (a) obtaining mRNA from a cell;
 - (b) contacting the mRNA so obtained with a probe comprising a nucleic acid molecule of claim 18 under hybridizing conditions; and
 - (c) detecting the presence of mRNA hybridized to the probe and thereby detecting the expression of the tumor suppressor by the cell.
37. A method for detecting expression of a tumor suppressor by detecting the presence of a tumor suppressor which comprises:
- (a) obtaining a cell sample from the subject;
 - (b) contacting the cell sample so obtained with an antibody of claim 26 under conditions permitting binding of the antibody to the tumor suppressor; and
 - (c) detecting the presence of the antibody so bound and thereby detecting the expression of the tumor suppressor.
38. The method of claim 37 for the detection of the expression of a tumor suppressor which has lost its tumor suppressor activity.
39. A method for diagnosing in a subject a predisposition to a tumor or to a disorder associated with the expression of a tumor suppressor allele which comprises:
- (a) isolating DNA from victims of the tumor or the disorder;
 - (b) digesting the isolated DNA of step (a) with at least one restriction enzyme;
 - (c) electrophoretically separating the resulting DNA fragments on a sizing gel;
 - (d) contacting the resulting gel with a probe comprising a nucleic acid molecule of claim 18 labeled with a detectable marker;
 - (e) detecting labeled bands on a gel which have hybridized to the probe as defined in (d) to create a band pattern specific to the DNA of victims of the tumor or the disorder;

- (f) preparing subject's DNA by steps (a) to (e) to produce detectable labeled bands on a gel; and
 - (g) comparing the band pattern specific to the DNA of victims of the tumor or the disorder of step (e) and the subject's DNA of step (f) to determine whether the patterns are the same or different and to diagnose thereby predisposition to the tumor or the disorder if the patterns are the same.
-
- 40. Use of an effective dose of a nucleic acid molecule of claim 1 or of any one of claims 11 to 17, or a nucleic acid molecule which is complementary to such a nucleic acid molecule for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.
 - 41. Use of an effective dose of a nucleic acid molecule of claim 18 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.
 - 42. Use of an effective dose of a vector of claim 19 or 20 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.
 - 43. Use of an effective dose of a polypeptide of claim 25 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.
 - 44. Use of an effective dose of an antibody of claim 26 for the preparation of a pharmaceutical composition for treating, preventing and/or delaying of reoccurrence of a disease in a subject.

45. The use of any one of claims 40 to 44 wherein the disease is a tumor or a neuronal disorder.
46. The use of claim 45 wherein the tumor is benign or malign and most preferably derived from endocrine or neuronal tissues, i.e. breast, lung, colon, intestine, stomach, prostate, testis, ovary, thyroid, pancreas.
47. A process for identifying compounds effective as antagonists/inhibitors or agonists/activators to a tumor suppressor comprising:
 - (a) contacting a cell which expresses the polypeptide of claim 25 with a compound to be screened; and
 - (b) determining if the compound inhibits or enhances activation of the tumor suppressor.
48. An antagonist/inhibitor or agonist/activator to the polypeptide of claim 25 or identified according to the method of claim 47.

Abstract

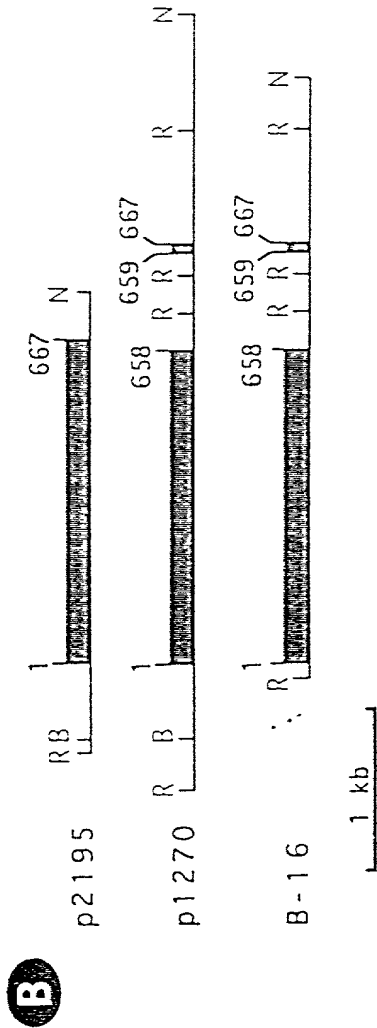
Described are novel proteins having the biological activity of a tumor suppressor protein and nucleic acid molecules coding for such proteins. Methods for the isolation of nucleic acid molecules encoding tumor suppressor proteins as well as nucleic acid molecules obtainable by said method are also provided. Further, vectors comprising said nucleic acid molecules wherein the nucleic acid molecules are operatively linked to regulatory elements allowing expression in prokaryotic or eukaryotic host cells can be used for the production of polypeptides encoded by said nucleic acid molecules which have tumor suppressor activity. Pharmaceutical and diagnostic compositions are provided comprising the nucleic acid molecules of the invention and/or comprising a nucleic acid molecule which is complementary to such a nucleic acid molecule. Described are also compositions which comprise polypeptides encoded by the described nucleic acid molecules which have tumor suppressor activity and/or an antibody specifically recognizing such polypeptides.

Figure 1 A

A

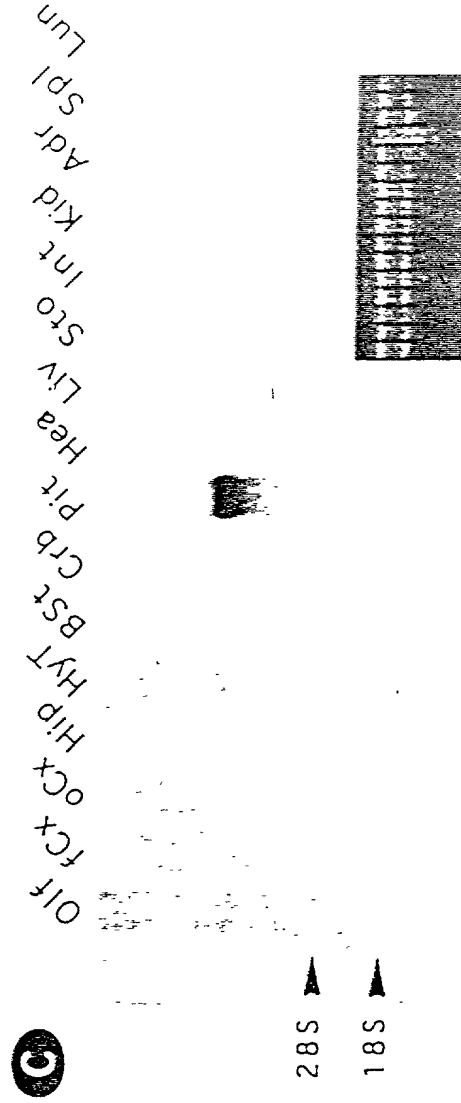
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561	QIQPQMQLPQ	LLPQLQPQQQ	PDPEPEPEPE	PEPEPEPEPE		600
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Figures 1 B and 1 C



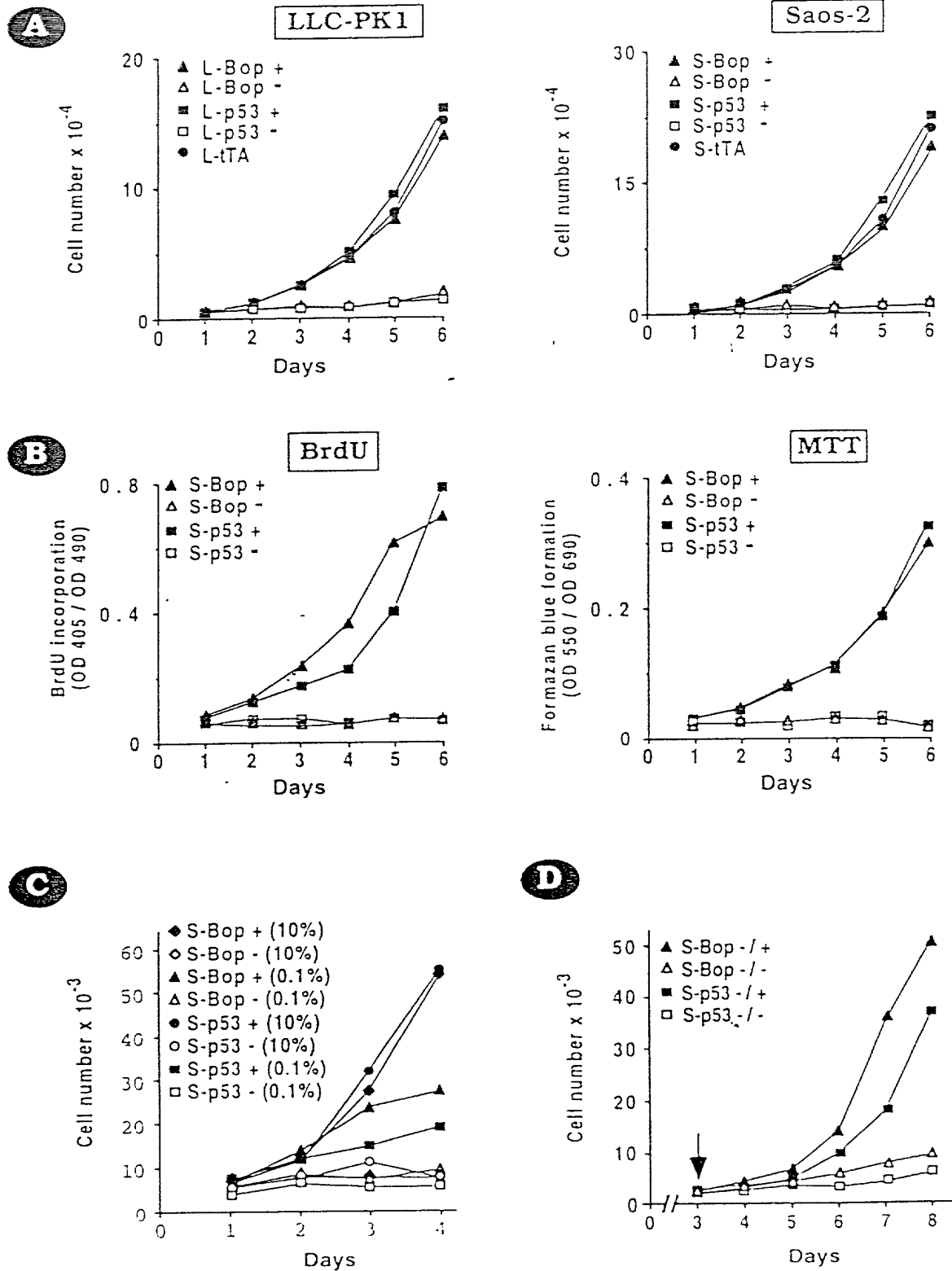
EXON Splice donor Splice acceptor EXON

5' ATA GCAGtgagtgctgtg.....gtttcttttcagGGA CTC 3'



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Figure 2



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Figure 3

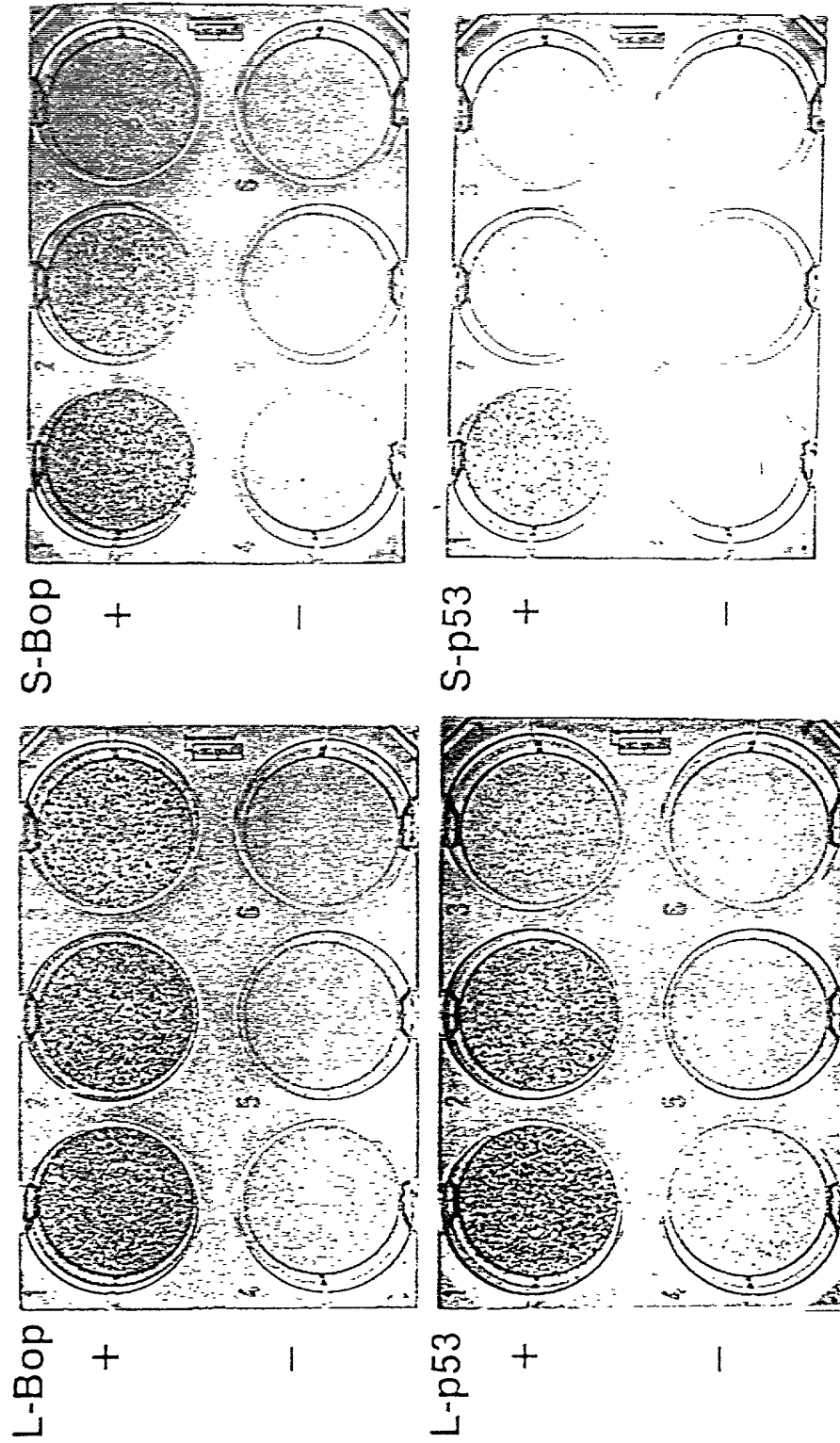
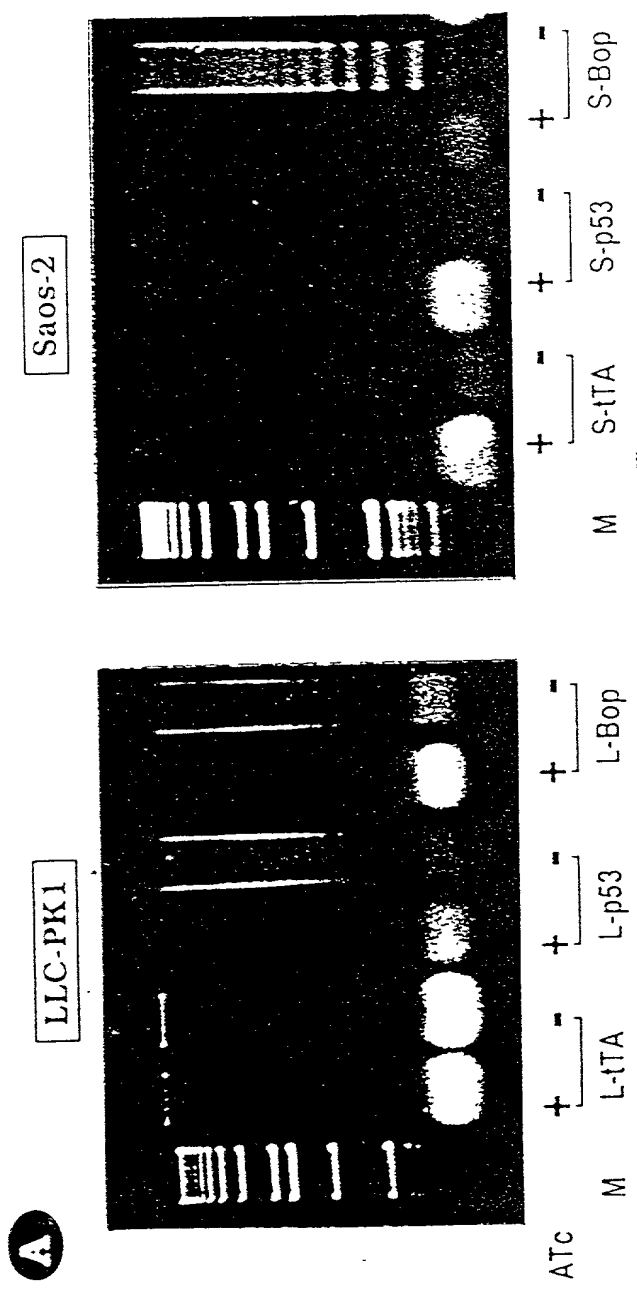
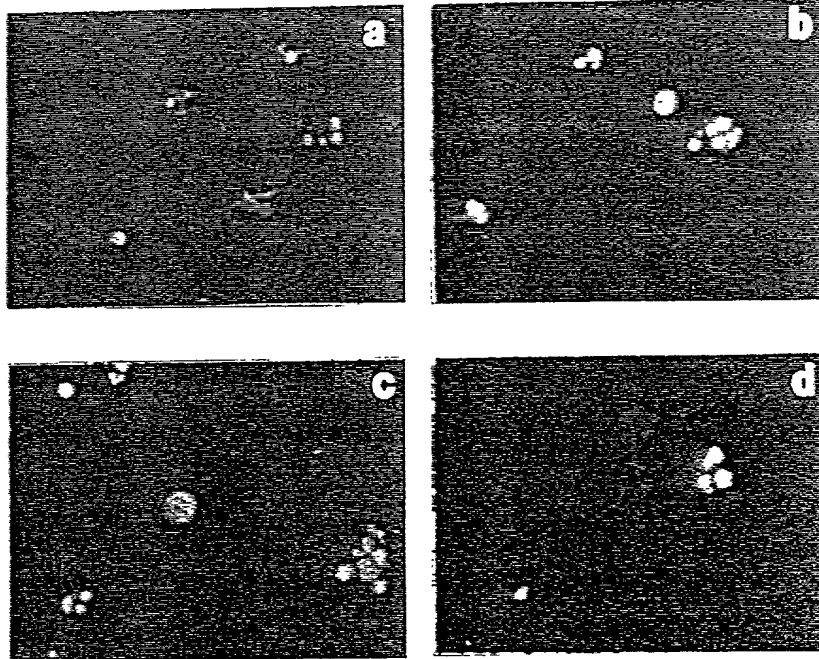
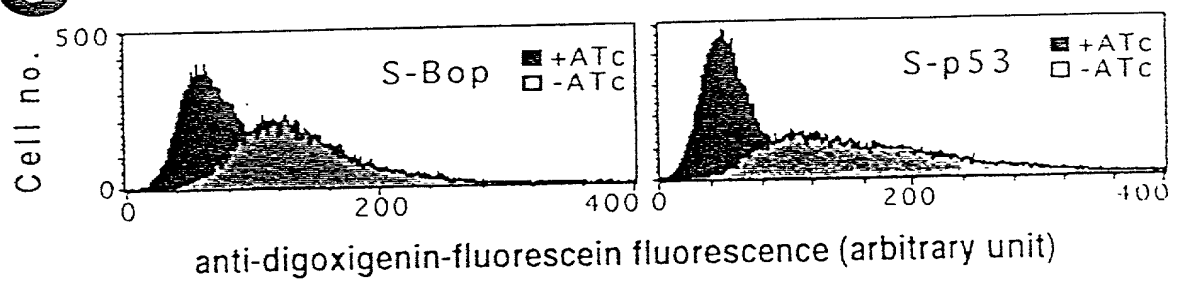


Figure 4 A



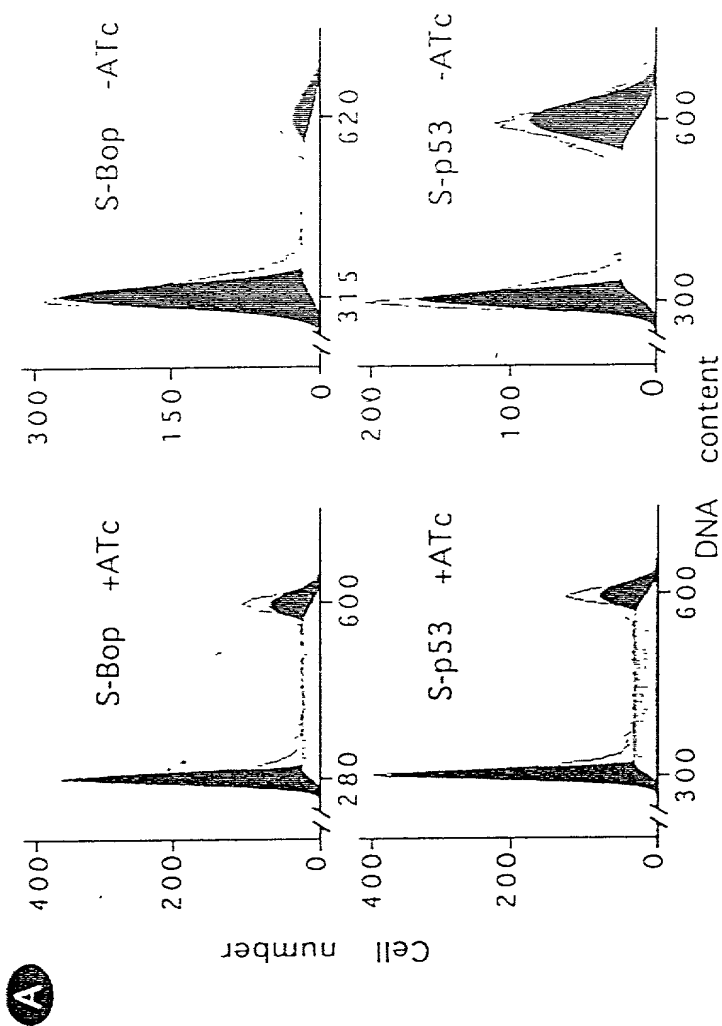
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Figures 4 B and 4 C

B**C**

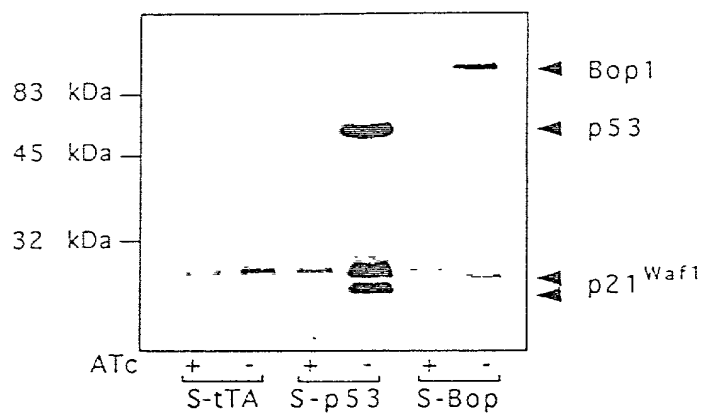
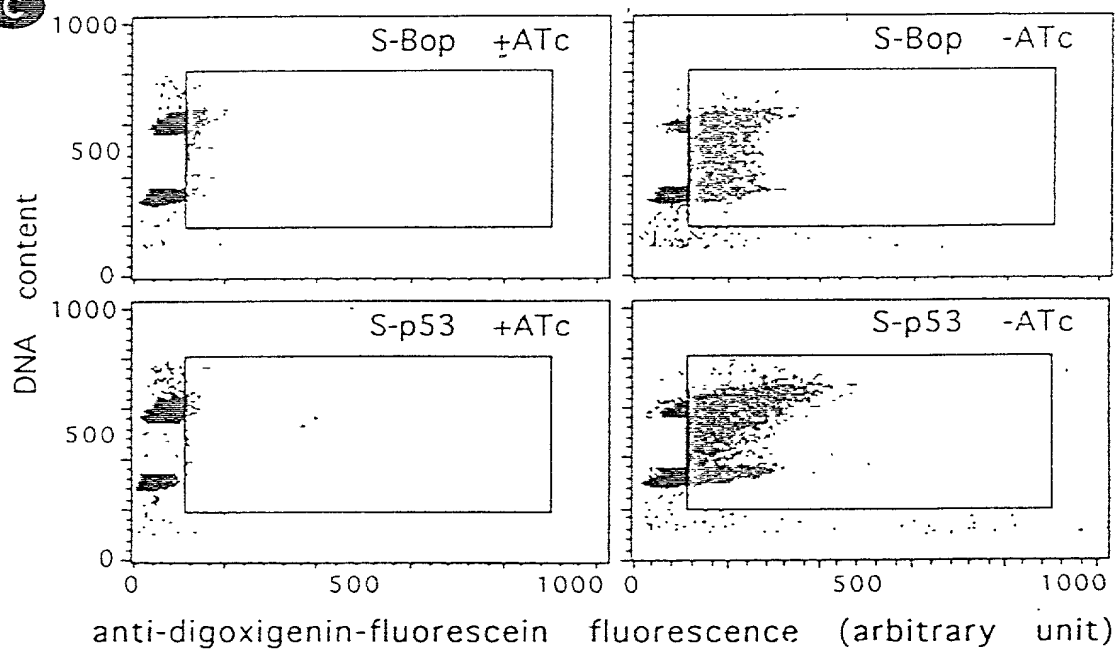
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Figure 5 A

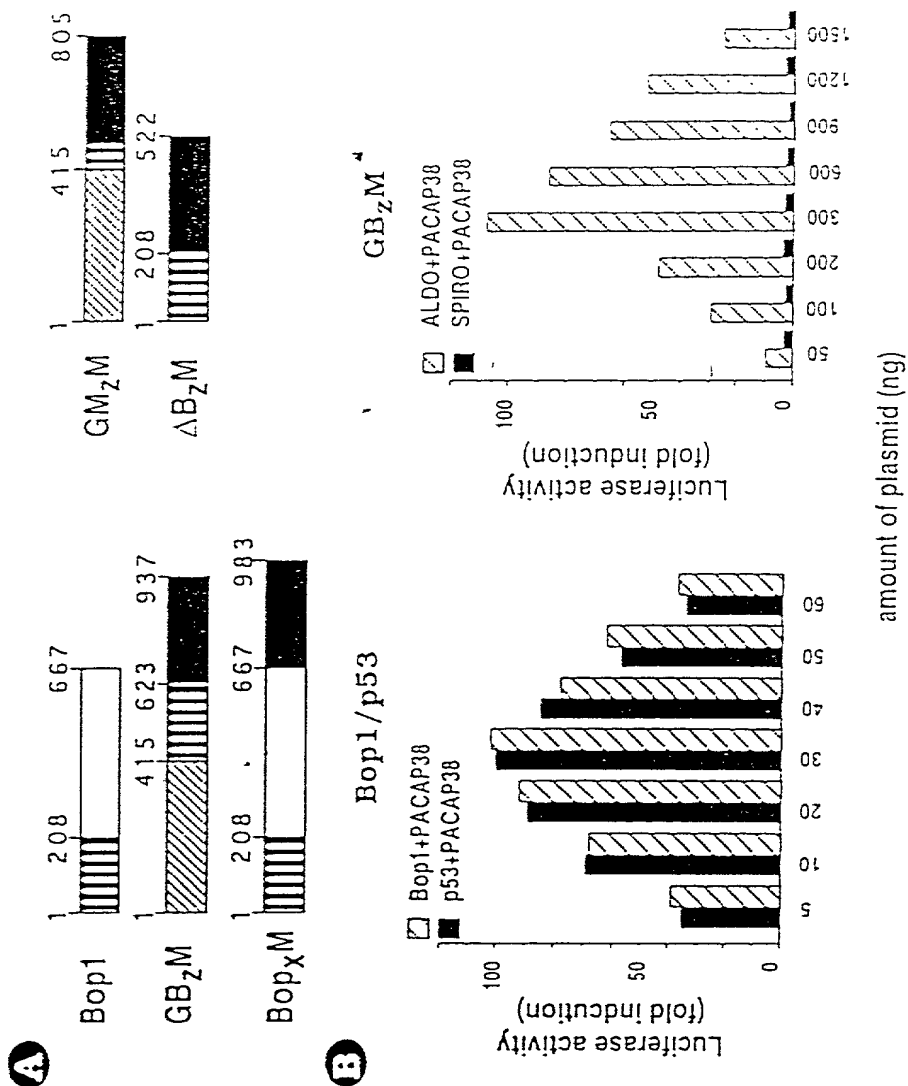


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Figures 5 B and C

B**C**

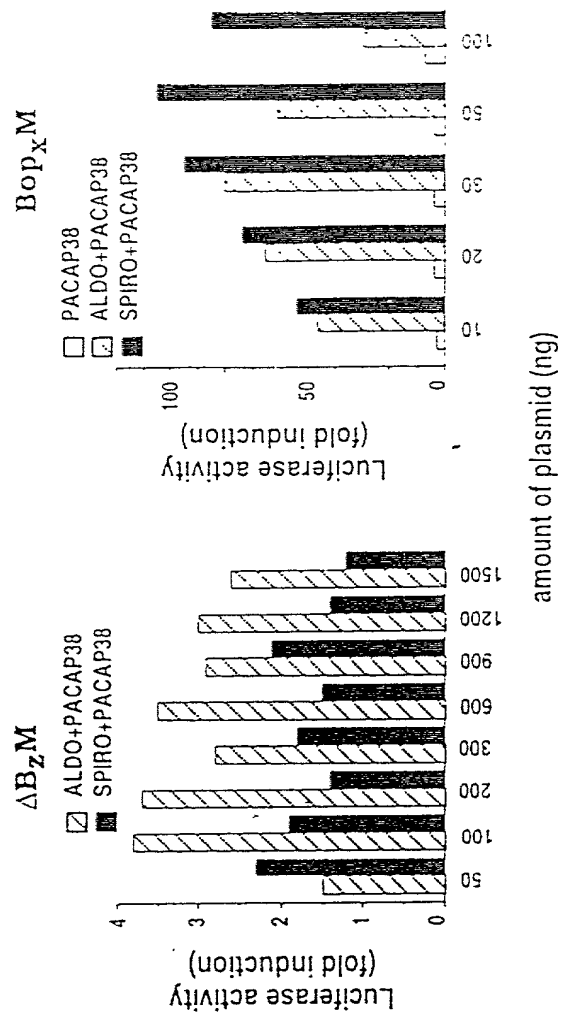
Figures 6 A and 6 B



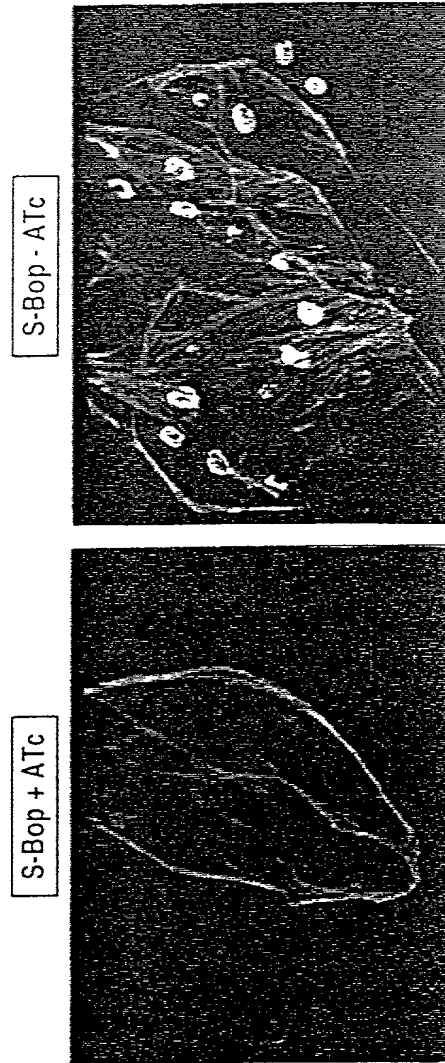
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Figures 6 C and 6 D

C



D



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(A)

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279  -----PESLASLHPSVSPGSPPE hZAC

401  IIPQEHKYNPVPTSYAPFVGMKADGKAFKMGFFEEFLQEPQAPLKFNECFEMMEGFGKVITLSKETILVDVAVNIAIF mZAC
303  IIPQEHKYSVPVTSYAPFVGMKADLKGFCNGLFEEFLQEPQAPLKFNECFEMMEGFGKVITLSKETILVDVAVNIAIF rLOT
297  E-LPNHKYNTTSTSYSPLASLPLKADTKGFCNTSLFEDLPLOEPOSOKLNPGFDLAKGNACKVNLKELPADAVNLTIE hZAC

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383  GSLEIISLLGFWQLPPPTIPNGEIVNSTIPVPEGEPLPHRIICLADQQPPPLPPPP-----PLPLPEPLPQPLPQPMQFQL rLOT
376  ASLDLSPLLGFWQLPPPATONTFGNSTIALGPGESLPHRLSCLGQQ----- hZAC

561  QIQPMQLPQLLPQLPQPPQPPDPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPEPE mZAC
458  QLQPP-----PQMPPQMLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQLPQL rLOT
423  ----- hZAC

641  EEEEEEAEEPQPEEAQIRVSAVNLCQPPPLPHIPIHIFTAGSNTAILPHFHHAFFR mZAC
531  EEAEEEEEPQPEEAQIRVSAVNLCQPPPLPHIPIHVFTAGSNTAILPHFHHAFFR rLOT
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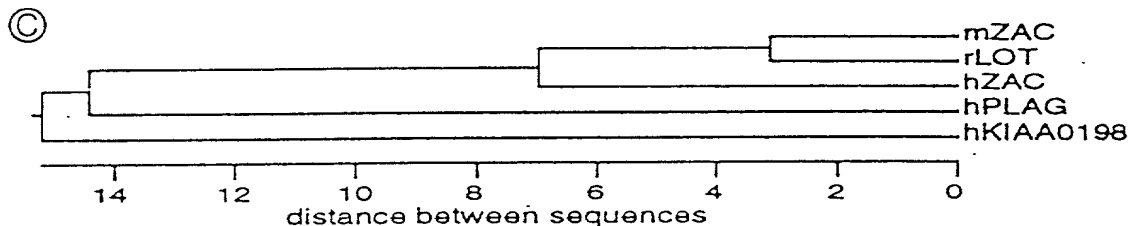
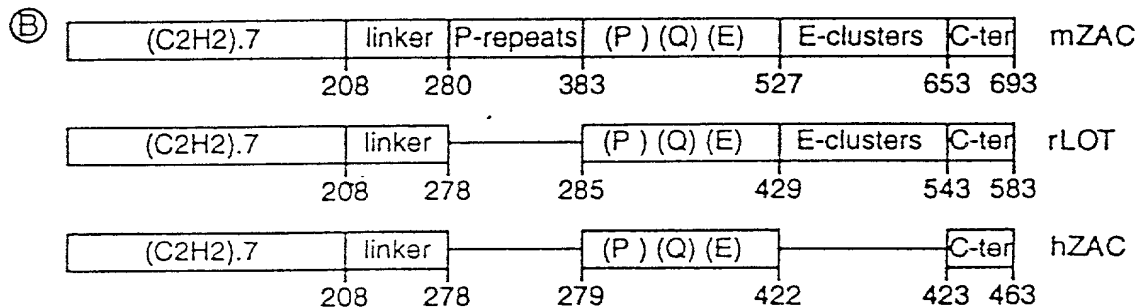


Figure 7

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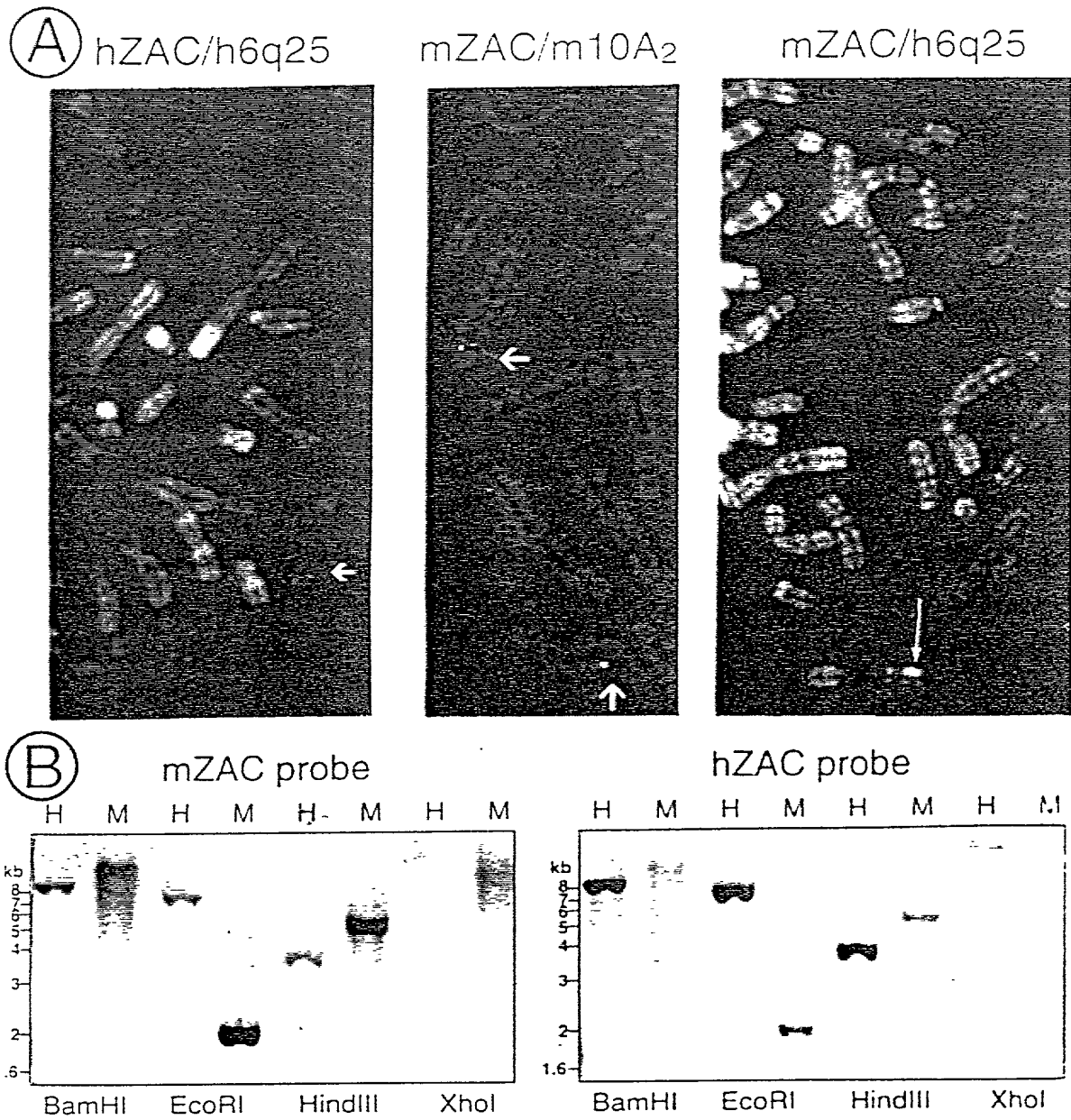


Figure 8

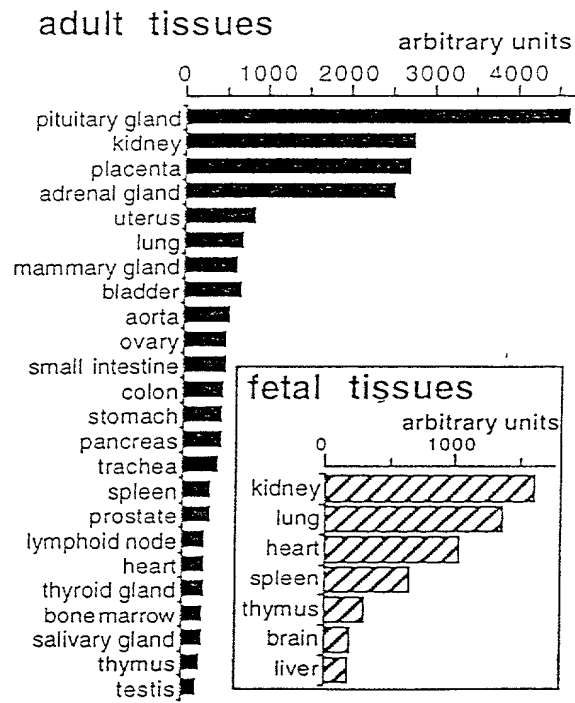


Figure 9

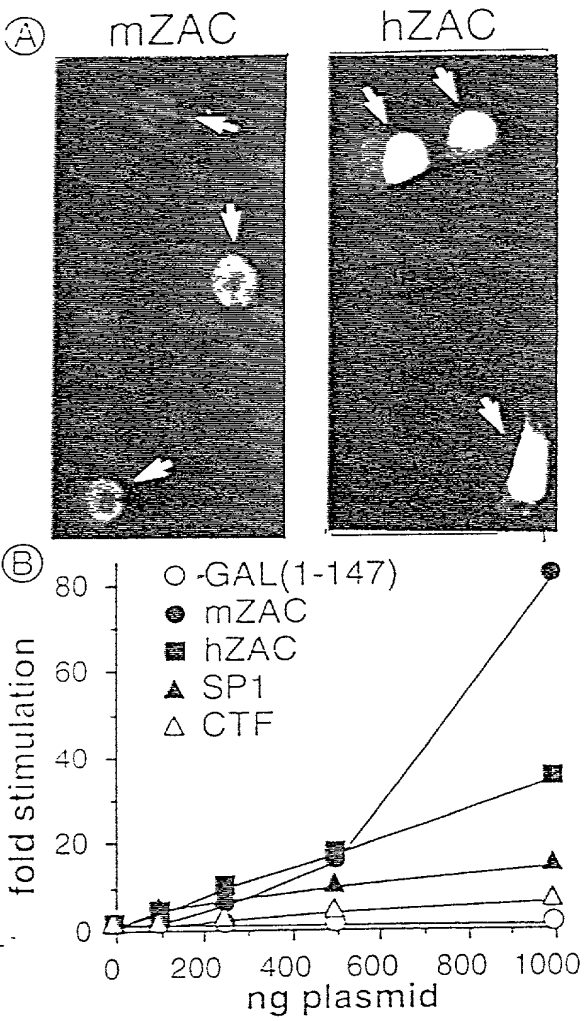


Figure 10

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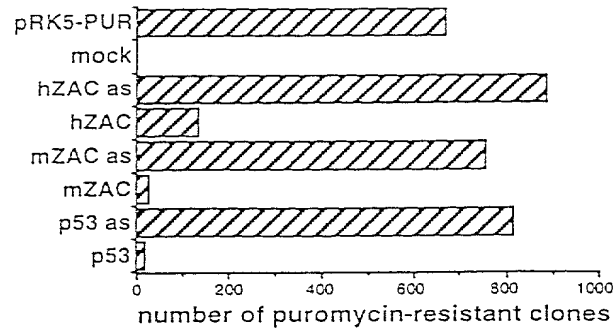


Figure 11

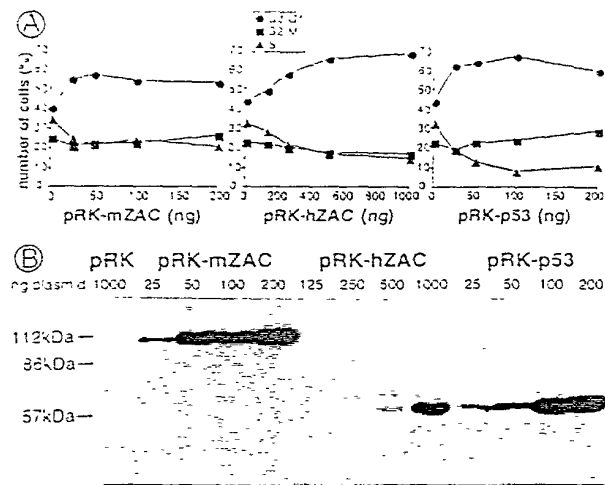


Figure 12

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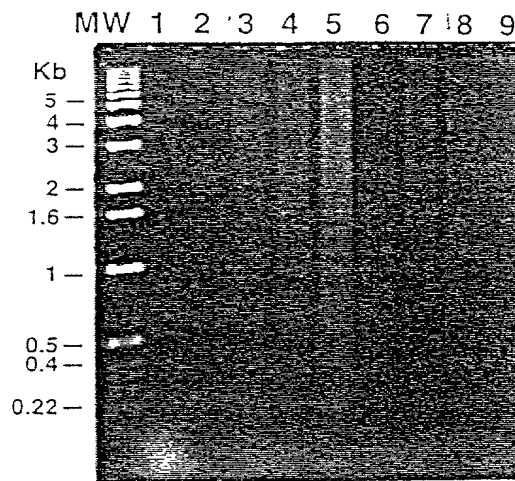


Figure 13

DECLARATION AND POWER OF ATTORNEY

As a below-named inventor, I hereby declare that:

My residence, post office address, and citizenship are as stated below next to my name.

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

NUCLEIC ACID MOLECULES CODING FOR TUMOR SUPPRESSOR PROTEINS
AND METHODS FOR THEIR ISOLATION

the specification of which:
(check one)

_____ is attached hereto.

X was filed on September 22, 1997 as
PCT International Application No. PCT/EP97/05198
Application Serial No. and enter the U.S. National Stage on
March 15, 1999
and was amended March 15, 1999
(if applicable)

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119 (a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International Application which designated at least one country other than the United States, listed below. I have also identified below any foreign application for patent or inventor's certificate, or PCT International Application having a filing date before that of the earliest application from which priority is claimed:

Prior Foreign Application(s)			Priority Claimed	
Number	Country	Filing Date	Yes	No
PCT/EP97/05198	European Patent Office	September 22, 1997	X	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____
_____	_____	_____	_____	_____

Declaration and Power of Attorney

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I hereby claim the benefit under Title 35, United States Code, Section 119(e) of any United States provisional application(s) listed below:

<u>Provisional Application No.</u>	<u>Filing Date</u>	<u>Status</u>
N/A		

I hereby claim the benefit under Title 35, United States Code, Section 120 of any United States Application(s), or Section 365(c) of any PCT International Application(s) designating the United States listed below. Insofar as this application discloses and claims subject matter in addition to that disclosed in any such prior Application in the manner provided by the first paragraph of Title 35, United States Code, Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56, which became available between the filing date(s) of such prior Application(s) and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
PCT/EP97/05198	September 22, 1997	pending as of March 15, 1999
08/718,661	September 23, 1996	patent 5,876,972

And I hereby appoint

17 John P. White (Reg. No. 28,628); Christopher C. Dunham (Reg. No. 22,031); Norman H. Zivin (Reg. No. 25,385); Jay H. Maioli (Reg. No. 27,213); William E. Pelton (Reg. No. 25,702); Robert D. Katz (Reg. No. 30,141); Peter J. Phillips (Reg. No. 29,691); Wendy E. Miller (Reg. No. 35,615); Richard S. Milner (Reg. No. 33,970); Albert Wai-Kit Chan (Reg. No. 36,479); Robert T. Maldonado (Reg. 38,232); Paul Teng (40,837); George M. MacDonald (Reg. No. 39,284); Richard F. Jaworski (Reg. No. 33,515); Elizabeth M. Wiecekowsky (Reg. No. 42,226); Pedro C. Fernandez (Reg. No. 41,741); and Gary J. Gershik (Reg. No. 39,992).

and each of them, all c/o Cooper & Dunham LLP, 1185 Avenue of the Americas, New York, New York 10036, my attorneys, each with full power of substitution and revocation, to prosecute this application, to make alterations and amendments therein, to receive the patent, to transact all business in the Patent and Trademark Office connected therewith and to file any International Applications which are based thereon under the provisions of the Patent Cooperation Treaty.

Declaration and Power of Attorney

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Please address all communications, and direct all telephone calls, regarding this application to:

John P. White

Reg. No. 28,678

Cooper & Dunham LLP
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New York, New York 10036
Tel. (212) 278-0400

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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